THE AMERICAN JOURNAL OF PATHOLOGY

VOLUME IX MARCH, 1933 NUMBER 2

THE MICROINCINERATION OF INTRANUCLEAR INCLUSIONS IN YELLOW FEVER *

E. V. COWDRY

(From the Anatomical Laboratory, Washington University, St. Louis, Mo.)

The method of microincineration offers a new means of approach for studies on the microchemistry of the nucleus. By burning away all the protein the hitherto insurmountable obstacle to the investigation of the mineral constituents is removed. Principal credit for the introduction of the technique is due to Professor A. Policard of Lyon. Improvements have been made by Scott [1] who, by studying the distribution of minerals in and about the chromosomes during cell division, has greatly extended our knowledge of this phenomenon. The same author [2] has found that the intranuclear inclusions caused by the submaxillary virus leave little or no ash, whereas the nucleoli of the affected cells are very rich in minerals. This has extended our ideas of the composition of the inclusions and has afforded a practical way of easily distinguishing them from nucleoli. It has also raised the question whether or not other nuclear inclusions in virus diseases behave in the same manner. The inclusions typical of yellow fever were selected for study because of previous experience with them by other methods (Cowdry and Kitchen [3]).

MATERIAL AND METHODS

The material consisted of pieces of liver, taken from a monkey sacrificed in a typical attack of yellow fever, fixed in absolute alcohol containing 10 per cent of neutral formalin, and very kindly sent to

* Aided by a grant from the Rockefeller Foundation for research in virus diseases. Received for publication November 16, 1932.

me by Dr. S. F. Kitchen of the International Health Division of the Rockefeller Foundation. The livers of monkeys not infected with the virus of yellow fever were used as controls.

It is unnecessary to give the technique of microincineration in detail for it has already been described in this Journal by Covell and Danks [4] and by Danks [5] in their reports on the cytoplasmic inclusions in rabies and in fowl-pox.

In this study serial paraffin sections were made 4 microns in thickness and mounted on slides. The sections on alternate slides were treated differently. The first were mounted in the usual fashion with egg albumin and were colored by Giemsa's stain. These served as controls, showing the appearance of the intranuclear inclusions as ordinarily seen. The second were mounted and incinerated in accordance with instructions by the investigators named, but for the guidance of others who may wish to repeat my observations two points may be noted.

Since nuclear material incinerates less readily than that of the cytoplasm, it is important to make sure that the incineration is complete. The following description relates to sections on slides placed in the electric oven and held between 95–100° C for ten minutes. The temperature was then gradually increased at the rate of approximately 100° C every five minutes to between 600 and 620° C, when they were allowed to cool and were covered. The ashes were examined in the dark-field and their appearance depended to some extent upon the light used and the optical equipment employed.

The source of illumination was a large-sized Spencer microscopic lamp (No. 394), fitted with a Projection Mazda, 500 W., 115 V. General Electric bulb. The distance between the filament and the mirror of the microscope was 18 inches. A piece of white frosted glass about 1 mm. thick was inserted at the end of the microscopic lamp near the mirror in place of the so-called "daylight glass." The light was accordingly rather yellow. Both the incinerated and the control-stained sections were examined with a Zeiss binocular monobjective microscope. For the former a Zeiss cardioid condenser was employed and for the latter a condenser of numerical aperture 1.40. The objective was a 3 mm. "X" with iris diaphragm, and paired compensating oculars X 10 were used for routine study.

Observations

In order that the results of microincineration may be viewed in proper perspective it is necessary to lead up to their consideration by reference to the appearance of the nuclei in the fresh unfixed condition and in stained preparations. This preliminary account will be based largely on a previous paper (Cowdry and Kitchen [3]). The steps in this transition from still living to fixed and stained, and to fixed and incinerated nuclei are illustrated diagrammatically in Plate 26 by three pairs of figures, 1 and 2, 3 and 4, and 5 and 6 respectively. The three in vertical column on the left represent normal nuclei viewed in these three states and those on the right, nuclei containing inclusions under corresponding conditions. It is obviously impossible to examine a nucleus in physiological saline; to fix, section, stain and re-examine the same nucleus; and finally to destain, incinerate and study it again for the third time. For the sake of clearness, however, the diagrams have been prepared as if this had been feasible, that is to say, nuclei of about the same size and showing approximately the same structure have been presented. The injured nucleus selected for this comparison represents a typical intermediate stage in the formation of inclusions, not the beginning or the end of the process. To save space on the plate, the cytoplasm has been omitted.

When the nucleus of a normal liver cell is examined in the living condition with high magnification and strong direct light, the nuclear membrane and the nucleolus can be distinguished easily, but the nucleoplasm appears to be of the same refractive index throughout its extent so that optical evidence is lacking of structural differentiation (Fig. 1). In the case of a nucleus altered by the virus of yellow fever, on the other hand, distinct nuclear inclusions can be seen in addition to the nucleolus (Fig. 2). These inclusions consist of clumps of tiny spherules of fairly uniform size. They occur usually to one side of the eccentrically placed nucleolus and separated from it and from the nuclear membrane by nucleoplasm in which no structure can be made out.

In fixed and stained preparations further details are noticeable in both normal and injured nuclei (Figs. 3 and 4). It is assumed that they are due to the accentuation by stains of the changes brought about by the fixative. In the normal (Fig. 3), the inner surface of

the nuclear membrane is no longer smooth and even, but is encrusted with basophilic material which is also found free in the nucleoplasm and in association with the nucleolus. This is represented in black. If the stain is suitably differentiated, a small amount of acidophilic material is also visible in the nucleoplasm and the nucleolus can be seen to have an acidophilic core. For contrast, these acidophilic components are depicted in gray. Of the two, the nucleolus certainly exists in approximately the same form in living cells, but the scattered acidophilic material in the nucleoplasm is probably, like the basophilic substance, a product of the coagulation of material originally distributed somewhat diffusely in it. The nuclei of cells injured by the virus of yellow fever, when stained in exactly the same way, present a very different picture (Fig. 4). Most of the basophilic chromatin is marginated irregularly on the nuclear membrane and comparatively little of it remains free in the nucleoplasm. The nucleolus is more rounded and has lost a good deal of the basophilic chromatin attached to it. The scattered particles of acidophilic chromatin are difficult to distinguish in the nucleoplasm. They may have completely disappeared or may be masked by the clumps of acidophilic particles making up the nuclear inclusions. The latter are very conspicuous structures and easily identifiable, but they are not so evenly rounded and uniform in size as when viewed in the fresh state.

In the incinerated normal nucleus (Fig. 5), the ash on the nuclear membrane is clearly that remaining from basophilic chromatin. The same interpretation is justified for the larger heavy masses of ash in the nucleoplasm and for the ash attached to the nucleolus. The few, small, feebly refractile clumps of ash in the nucleoplasm may represent the remains of the scattered acidophilic chromatin. The identification of the large, dense, ovoid mass of ash with the acidophilic nucleolus is also evident. In the injured nucleus (Fig. 6), incineration gives a heavy ash on the nuclear membrane, which remains from the burning of marginated basophilic chromatin (Fig. 4), which in turn is probably a coagulum of more diffusely distributed substance brought about by the fixative, as it is not visible in the living condition (Fig. 2). The nucleolus yields a very compact deposit of white ash which is similar in shape to the acidophilic core (Fig. 4). About this deposit is a lighter ash which remains from the basophilic chromatin associated with the core. In the nucleoplasm

there are a few scattered ashes as residues of depleted chromatin in this situation, but it is unsafe to hazard an opinion as to whether this chromatin was basophilic or acidophilic in nature. No definite traces are to be seen of the inclusions which were a prominent feature of the altered nucleus when observed in the living state (Fig. 2), and after fixation and staining (Fig. 4).

The description may now be extended to include an account of the liver cells reacting to the virus from the first to the latest stages, as seen after microincineration, and in alternating sections colored by Giemsa's stain. The figures on Plate 27 were drawn at the level of the table with objective "X," compensating oculars X 20, and camera lucida. They illustrate the cytoplasmic ash as well as the nuclear ash.

The cell membrane shows up in the dark-field as a glistening line of white ash. The line is, however, not of equal thickness throughout. It is made up of small clumps of ash which are unevenly spaced. Between such clumps no traces of the membrane can be made out although alternate stained sections of corresponding cells indicate that the membrane is continuous. This may mean: (1) that during life the mineral constituents are irregularly distributed with reference to the membrane, or (2) that the clumping is an expression of the coagulation that occurs when the tissue is fixed, or (3) that it takes place during the process of incineration. In later stages involving necrosis, rupture of the membrane is indicated by lines of ash pointing in different directions (Fig. 14). The cytoplasm leaves a greater residue in the central and peripheral zones of the lobule than in the intermediate zone which is most affected by the virus. Figures 7–9 illustrate cells in the peripheral zone and Figures 10–14 in the intermediate. It will be observed that for similar volumes the cytoplasm shows distinctly less mineral than the nucleus, and also that it disappears more rapidly as necrosis advances.

The nuclear membrane is likewise outlined by an interrupted ash deposit. In Figures 7–9 its contours can, however, be clearly made out. In the cells represented in Figures 10–14 the nuclei are shrunken and to some extent fragmented. Figure 10 shows a heavy accumulation of ash in or on the lower part of the membrane as it is illustrated. No remains of the upper part are to be seen. The ash is also very marked in Figure 11, in which the nuclear membrane appears to be breaking up. Further shrinkage is indicated in Figures 12 and 13,

while only traces of the membrane remain in the cell shown in Figure 14.

The nucleolus leaves a much heavier and more compact ash than any other constituent of the liver cell. In Figure 7, which illustrates but slight injury, it can be seen to be made up of a dense core, on the upper left margin of which some less compact ash can be observed. Traces of this less dense ash are also revealed in the binucleated cell represented in Figure 9. In Figures 8, 10 and 12, however, only the spherical core can be distinguished. This core evidently remains without noticeable change, while marked alterations occur in the cell membrane and in the cytoplasm, as well as in the nuclear membrane, but it usually disappears before all traces of the nuclear membrane are lost.

The nucleoplasm is of special interest because it is in it that the inclusions can be easily observed in fresh preparations and in fixed and stained ones. After incineration of nuclei only slightly injured by the virus, as judged by their plump appearance and the relatively normal cytoplasmic ash, there is no accumulation of ash detectable, by the methods used, in the intermediate zone of nucleoplasm where we have reason to believe early inclusions existed (Fig. 7). The scattered particles of ash which persist do not correspond with the inclusions seen in similarly injured cells in alternating stained sections. They tend to be rod-like and angular, vary considerably in size and are often massed near the ash of the nucleolus and of the chromatin associated with the nuclear membrane, leaving the intermediate zone of nucleoplasm clear. In all probability they are the residue of the small amount of chromatin remaining in the nucleoplasm. Figure 8 illustrates a stage, if anything, slightly more advanced. To the left of the compact ash of the nucleolar core can be made out two very faint spherical masses of ash which occupy the position usually taken by the inclusions. Though they are somewhat larger than the individual particles which make up the inclusions it cannot be asserted positively that they are not inclusions. Such appearances are, however, very rare. The conclusion is justified that in the vast majority of cases the inclusions leave no mineral residue detectable after the incineration specified, and with the illumination and optical system employed. With less complete incineration microscopically visible residue might remain, consisting partly of organic material. Figures 10–14 show much more severe

nuclear injury. In Figures 10 and 13 a little mineral is illustrated in the nucleoplasm, which probably represents the last remaining chromatin. Examination of Figures 11, 12 and 14 shows nucleoplasm which is apparently devoid of ash (except of the nucleolus, Fig. 12), although the alternating control stained sections reveal the fact that nuclei in these conditions are packed with acidophilic inclusion material. Colored illustrations of nuclei in correspondingly advanced stages leading to karyorrhexis (Fig. 14) are given by Cowdry and Kitchen [3] in their Figures 22–24 and 42, inspection of which shows masses of characteristically particulate, acidophilic material partly enclosed by marginated basophilic chromatin which is represented in these incinerated nuclei by dense ash.

The mineral residue, which thus far has been referred to simply as "ash," consists of oxides of various elements. Most of it is amorphous and not crystalline, probably because some water vapor unavoidably gained access to it after incineration before it could be covered and sealed with paraffin. Those who have had experience in microincineration are of the opinion that calcium can be identified in the dark-field by its flat white appearance, as well as by the forming of gypsum; sodium by its bluish white sheen *; iron by its faint dull red color; and silica by its birefringence in polarized light because it alone is crystalline. Scott [6] gives a critique of the evidence. Colors are difficult to identify and measure and are conditioned to some extent by the light used in making the observations. Generally speaking, the ash associated with the cell membrane of liver cells in this study and remaining from the basophilic chromatin and nucleolus was creamy white. A tinge of red was sometimes seen in that of the basophilic chromatin and especially of the nucleolus. This was not, however, by any means constant. That it may represent iron is indicated by the application of the Bensley-Macallum test which demonstrated the presence of some iron in these substances and none in the nuclear inclusions. Cowdry and Kitchen [3] found that the inclusions gave a negative Feulgen reaction for thymonucleic acid, whereas the chromatin and nucleolus yielded positive ones. We have to recall in this connection that the nucleolus in the liver cells of monkeys is more accurately described by the

* Mason's paper on "transmitted structural blue" (*J. Phys. Chem.*, 1931, **35**, 73–81) indicates that this color may be caused by the physical and not the chemical properties of the ash.

term amphinucleolus, for it contains both acidophilic and basophilic material. The cytoplasmic ash was often, but again not invariably, more faintly bluish white than that of other parts of the cell. Traces of bluish ash were occasionally noted also in the nucleoplasm. Doubly refractile material was not found in the nuclei, although a little was seen along the course of the sinusoids and in the periportal connective tissue.

On the quantitative side we obviously cannot be sure that *all* of the mineral substances are completely immobilized at precisely the places that they occupy in the living cells because coagulation by the fixative, though rapid, is not instantaneous. The possibility exists, therefore, that some of the mineral may have been displaced and may even have left the cells. Policard and Okkels [7] have calculated that in some cases the loss may be 10–14 per cent. In a recent paper Gersh [8] mentions this problem of fixation and makes the following statement: "The ash observed after microincineration may and probably does represent a distribution of inorganic constituents which does not obtain during life. That the distribution has been altered will be demonstrated at another time." It would have been more helpful had he presented the evidence with the criticism.

In 1924 Policard, Noël and Pillet [9] reported very briefly and without illustrations the effect of different diets on the ash from sections of the livers of mice. After feeding only sugars the central parts of the lobules appeared to be much richer in ash than the peripheral (periportal). The former were much more quickly incinerated than the latter. Following a strictly protein diet the distribution of ash was uniform throughout the lobule and it was even less in amount than that in the peripheral parts of the lobules of the mice given sugar. The incineration was more difficult, the nuclei in particular remaining black for a long time. Finally, on a diet made up altogether of fat, the distribution of ash was very even and the incineration was easier than after the protein diet, and more difficult than after the feeding of sugar. No interpretation of these findings was offered.

Later, in 1931, Noël, Pigeaud and Millet [10] made a study of the mineral content of the human fetal liver at different ages by incineration in bulk and by microincineration. They found by the first method that it increased markedly between the second and fourth

month, then remained fairly constant, to increase sharply again in the last two months. Apparently the distribution of the ash was not studied in detail by microincineration for no reference is made of nuclear or cytoplasmic ash, let alone parts of these structures, but distinction was made of the residues of the periportal connective tissue, the blood vessels and their contents. Red ash corresponding to iron was observed, particularly in fetuses over 4 months, but not in relation to blood vessels. All the ash was positive for calcium by the gypsum test.

The inadequacy of our data regarding the physiological significance of the ash normally present in the liver does not detract from objective descriptions of changes in amount in pathological states. In order, however, to have some base line as to the variability or uniformity in the ash of liver cells I have examined incinerated sections of the liver of a man 44 years of age who died of double pneumonia, and of a cat, 3 rabbits, 1 guinea pig and 1 rat which were apparently normal. All of them left ash which corresponded with that of *Macacus rhesus* in the following particulars: (1) The ash of similar topography to the cell membrane was creamy white. (2) The cytoplasmic ash was by contrast pale bluish white, but the ratio of white to bluish ash in it was not constant in all the species. (3) The ash in or applied to the nuclear membrane was creamy white, as was also most of the ash in the nucleoplasm as well as that of the nucleolus. (4) Occasionally a little pale bluish white ash was noted in the nucleoplasm. (5) Of all elements in the cell the nucleolus left the heaviest and most dense ash, but the nucleoli were more pronounced in some species than in others. (6) Dull red ash was of rare occurrence but was detected in some cases in both nucleus and cytoplasm. (7) No ash birefringent in polarized light was observed either in the nucleus or the cytoplasm of liver cells. (8) Per unit volume the ash of the nucleus was more marked than that of the cytoplasm.

Discussion

From the foregoing description it is evident that the intranuclear inclusions which appear in the liver cells of monkeys under the influence of the virus of yellow fever generally differ both from the nucleolus and from basophilic chromatin in the absence of detectable amounts of mineral matter. This observation supplements Scott's

discovery that the intranuclear inclusions caused by the submaxillary virus in guinea pigs are likewise ash-free.[2]

Since inclusions which are characteristic in the sense that they resemble the type inclusions of herpes have other microchemical properties in common in virus III disease, varicella, submaxillary disease and yellow fever (Cowdry,[11] Cowdry and Kitchen [3]), it will be interesting to ascertain if this absence of detectable mineral matter also runs through the series. If so, the technique of microincineration will afford a ready means of comparing less typical inclusions with nucleoli in the same and adjacent cells. The inclusions in Borna disease, poliomyelitis (Covell [12]), and Rift Valley disease (Daubney, Hudson and Garnham [13]) belong in this category, although the evidence may be satisfactory that they represent some sort of response to the several viruses. It is not unlikely that microincineration might bring data which would settle the status of the inclusions reported by Findlay [14] in mice, with the suggestion that they may be of nucleolar nature.

In view also of the microchemical similarity of the typical inclusions named, it is natural to inquire how far theories advanced to explain them are applicable to those of yellow fever.

In 1923 Goodpasture and Teague [15] made the following statement: "The constancy with which the intranuclear bodies occur and the characteristic morphological and tinctorial properties which they present in experimental lesions of herpes febrilis in rabbits have convinced us that they represent, as Lipschütz claims, the presence and growth, within the nucleus, of the specific virus of the disease. It seems probable that the virus itself may be obscured by a mantle of nuclear material which gives to the inclusions their usually homogeneous appearance and acidophilic staining quality. . . ." In another place they say that "the intranuclear bodies are essentially masses of virus." Later on Goodpasture [16] reported: "It seems evident, however, that the material which constitutes the 'inclusion' may partly at least be composed of coagulated nucleoplasm which may impart the acidophilic staining property of the inclusions. It is to be noted, however, that when the minute granulations are discrete enough to be recognized as such they stain faintly basophilically, whereas the precipitate from the nucleoplasm of normal cells is more acidophilic. They are to be regarded at present as elementary bodies taking part in the structure of the

herpetic inclusions." My own studies [11, 17] on intranuclear inclusions in herpes have not brought evidence of the existence of two kinds of material in them which could be interpreted in this way.

There is no reason to believe that the yellow fever inclusions are composed of two sorts of substances. Like those in herpes they are acidophilic and uniformly so, but the color naturally depends to some extent upon the technique employed and the extent of differentiation, or the degree of extraction of the dyes. No traces could be found of minute granulations staining faintly basophilically, which could be regarded as elementary bodies. All the available data indicate that their reactions to various tests are total responses in which all of their substance is involved. Thus they completely disappear when treated in the fresh condition with dilute acetic acid (Cowdry and Kitchen [3]); they apparently leave no recognizable ash and the Feulgen reaction is wholly negative, also the test for masked iron, and so on. Because as far as we can tell at present the material is not a mixture of easily recognizable substances, it does not follow that it is a single chemical compound. What concerns us is that it cannot be resolved into nuclear material plus elementary bodies by standard tinctorial methods, or by those microchemical tests which have been employed. We have to consider not only the evidence but also the balance of probability. Though we cannot *see* elementary bodies or virus within the affected nuclei, we cannot deny their presence. The burden of proof is on those who make the statement that they occur therein.

The elementary bodies are supposed to be tiny microörganisms which invade the nucleus and multiply within it. Pinkerton and Hass [18] have recently shown that the *Rickettsiae* of Rocky Mountain spotted fever behave in just this way in tissue cultures of the membranous exudate of the scrotal sac of an infected guinea pig. This is an important extension of Wolbach's observation that the same *Rickettsiae* are sometimes intranuclear in ticks.[19] The authors describe these intranuclear *Rickettsiae* in cultures fixed in Regaud's fluid, sectioned at about 7 microns and colored by Giemsa's stain. "The organisms varied in their staining reactions from blue through the purples to red, depending upon the degree of differentiation, but never attained quite the bright red of the granules of polymorphonuclear leucocytes." They were arranged in clusters in the nucleoplasm, separated from the nuclear membrane by definite halos. "In

some cases the individual organisms of a group could not be resolved and the clusters appeared as hyaline masses." Pinkerton and Hass say that it seems unnecessary to comment on the resemblance of these clusters, "especially when imperfectly fixed and stained, to certain of the structures of unknown nature found within cells in the so-called virus diseases. . . . It therefore seems not improbable that some of the unresolved intranuclear structure now classed as inclusion bodies may be of a similar nature."

Unfortunately I have not seen the preparations of Pinkerton and Hass, but they are illustrated by an excellent colored plate. The only cell shown in which there is a resemblance between the clumped *Rickettsiae* in the nucleus and intranuclear inclusions caused by viruses is the one in the upper left-hand corner of the plate. In it the mass has the hyaline appearance to which they call attention and it is surrounded by a halo, but they state that their sections were cut 7 microns in thickness. Had they been thinner and had the comparison with typical intranuclear inclusions been more direct after fixation in the same fluid and after mounting together on the same slide, I question whether this similarity would hold. But more important are the stages in clumping of the *Rickettsiae* and in development of the inclusions. They are entirely different. The organismal nature of the *Rickettsiae* is so clear that it cannot be mistaken, whereas exhaustive examinations made by numerous investigators of the stages in development of inclusions typical of virus action have never revealed the multiplication, clumping and fusion of bodies even remotely resembling the *Rickettsiae*.

Moreover, the intranuclear clumping of *Rickettsiae* is a very exceptional occurrence and there is an element of weakness in basing thereon an interpretation of intranuclear inclusions which are certainly very widely distributed. The authors state that it ". . . is the first instance in which a definite microörganism has been shown to be parasitic in clusters in the nuclei of mammalian tissues." But they have apparently only found them in tissue cultures of the said tissues. Evidence that they occur in the actual tissues of animals in experimental Rocky Mountain spotted fever is conspicuous by its absence. Wolbach [19] failed to find them, though he discovered them in ticks, and Nicholson,[20] working in my laboratory, searched for them without success. That they do become intranuclear in tissue cultures and not in the animals may be conditioned by some

alteration in the cells occasioned by their removal from the body and their growth *in vitro*.

If the inclusions caused by the yellow fever virus do contain microörganisms akin to bacteria as elementary bodies, one would expect them, in common with most bacteria, to possess a good deal of mineral matter. The observation that the intranuclear inclusions in yellow fever do not possess sufficient mineral to leave a recognizable ash, when treated by the method specified in this paper, does not support the idea that bodies of this type occur in large numbers in these inclusions. Scott's discovery [2] that the nuclear inclusions caused by the submaxillary virus in guinea pigs are likewise completely incinerated, leaving no ash, places them in the same category. But not until the new technique has been carefully applied to other intranuclear inclusions associated with virus action will a statement be justified as to how general is this attribute.

The fact that some incitants of disease, which were thought to be ultravisible and were looked upon as viruses, have been proved to be tiny microörganisms should not influence our point of view more than the situation warrants. Among them, the *Rickettsiae* of heartwater [21] and psittacosis [22] may be mentioned. It may be significant that despite the most painstaking search there is not a single instance in which a virus which brings about the development of an intranuclear inclusion has been demonstrated to be organismal. Yet this is only one of several reasons why those who have been concerned chiefly in the study of viruses which produce intranuclear inclusions should vigorously question the validity of the assumption that this particular group of viruses is organismal. Viruses which cause the development of cytoplasmic inclusions are on a different basis. Some of them may be true microörganisms and others not, the probability hinging, in the absence of crucial information, upon their chemical and physical properties, their resistance to agents which are lethal to known forms of life, and so on. The investigations of Goodpasture and Woodruff [23, 24] on the cytoplasmic inclusions in fowl-pox suggest that they contain minute organisms which are the etiological agents of the disease, but fall short of being entirely convincing. It is worthy of note that these inclusions and the Negri bodies are the only ones that have been studied by the method of microincineration.[4, 5] Both differ from intranuclear inclusions by yielding an abundant ash. This does not signify that

they are necessarily organismal; for the evidence against this interpretation of the Negri bodies is convincing and many cellular components, which are certainly not made up of invading organisms, leave an ash, but it is consistent with the view that they may be composed of bacteria-like parasites.

Since there is no direct evidence, or reason to suppose from doubtful analogy, that the inclusions typical of the yellow fever reaction do contain any organisms in the form of elementary bodies or in any other state, we may now pass to a consideration of the possibility that they consist in whole or in part of "virus," meaning thereby what the word implies, namely, a poison. Notwithstanding the observation that virus increases in amount in localities where the inclusions are developing or have formed, to contend that this topographic association in yellow fever, herpes or any other disease where it obtains, indicates that the inclusions are masses of virus is to take an altogether indefensible position. It cannot be denied that the virus may enter the nuclei because the cells have clearly been injured and their permeability altered. In the initial stages of the reaction the nuclei are sometimes a little swollen, pointing to the intake of fluid. Moreover, the virus is a substance the constituent particles of which in a watery environment are extremely small, so that it may conceivably pass through membranes relatively freely. But the penetration of virus into the nuclei and its concentration in them in the form of inclusions microscopically visible has simply not been proved. While we cannot recognize the yellow fever virus by any specific chemical test in highly potent filtrates, the possibility of doing so in the cell, itself, is remote.

Summary

1. In preparations of uninjured liver cells of the monkey made by microincineration, as specified in the foregoing pages, the nuclear ash corresponds closely in position with materials seen in the fresh cells, as well as in fixed and stained preparations. The nucleolus — easily recognizable in fresh cells by its position, shape and refractive index — is found to be amphophilic in fixed and stained specimens and to yield a very heavy, sharply localized ash after incineration. Chromatin, which is not visible as such in the still living cell but can be observed after fixation and staining in the form of basophilic

substance scattered in the nucleoplasm and applied to the nuclear membrane, also leaves a mineral residue which is rather less dense.

2. Marked alterations occur in nuclei reacting to the virus of yellow fever and in which nuclear inclusions are developing. The changes in size and shape of the nuclei, in the basophilic chromatin and in the nucleolus, described by Cowdry and Kitchen in stained preparations, can be followed with almost equal precision in the incinerated specimens because parallel modifications occur in the mineral residue. But the nuclear inclusions, pathognomonic of the disease, although conspicuous features of the fresh and fixed and stained preparations, cannot be studied in incinerated specimens for they yield little or no ash. They therefore differ from the nucleoli and from basophilic chromatin in the same way that Scott observed in the case of nuclear inclusions caused by the action of the submaxillary virus in guinea pigs.

REFERENCES

1. Scott, G. H. The disposition of the fixed mineral salts during mitosis. *Bull. d'histol. appliq. à la physiol.*, 1930, **7**, 251–256.
2. Scott, G. H. Sur la localisation des constituants minéraux dans les noyaux cellulaires des acini et des conduits excréteurs des glandes salivaires. *Compt. rend. Acad. d. sc.*, 1930, **190**, 1073.
3. Cowdry, E. V., and Kitchen, S. F. Intranuclear inclusions in yellow fever. *Am. J. Hyg.*, 1930, **11**, 227–299.
4. Covell, W. P., and Danks, W. B. C. Studies on the nature of the Negri body. *Am. J. Path.*, 1932, **8**, 557–571.
5. Danks, W. B. C. A histochemical study by microincineration of the inclusion body of fowl-pox. *Am. J. Path.*, 1932, **8**, 711–716.
6. Scott, G. H. The localization of mineral salts by microincineration in the cells of some mammalian tissues. (In press.)
7. Policard, A., and Okkels, H. Localizing inorganic substances in microscopic sections. The microincineration method. *Anat. Record*, 1930, **44**, 349–361.
8. Gersh, I. The Altmann technique for fixation by drying while freezing. *Anat. Record*, 1932, **53**, 309–337.
9. Policard, A., Noël, R., and Pillet, D. Etude histochimique des variations de la teneur en cendres du tissu hépatique suivant divers régimes. *Compt. rend. Soc. de biol.*, 1924, **91**, 1219–1220.
10. Noël, R., Pigeaud, H., and Millet, P. Étude histochimique de la teneur en matières minérales fixes du foie chez le foetus humain aux différents âges. *Bull. d'histol. appliq. à la physiol.*, 1931, **8**, 27.
11. Cowdry, E. V. The microchemistry of nuclear inclusions in virus diseases. *Science*, 1928, **68**, 40–41.

12. Covell, W. P. Nuclear changes of nerve cells in acute poliomyelitis. *Proc. Soc. Exper. Biol. & Med.*, 1929–30, **27**, 927–929.
13. Daubney, R., Hudson, J. R., and Garnham, P. C. Enzoötic hepatitis or Rift Valley fever. An undescribed virus disease of sheep, cattle and man from East Africa. *J. Path. & Bact.*, 1931, **34**, 545–579.
14. Findlay, G. M. Intranuclear bodies in the liver-cells of mice. *Brit. J. Exper. Path.*, 1932, **13**, 223–229.
15. Goodpasture, E. W., and Teague, O. Experimental production of herpetic lesions in organs and tissues of the rabbit. *J. Med. Res.*, 1923, **44**, 121–138.
16. Goodpasture, E. W. Intranuclear inclusions in experimental herpetic lesions of rabbits. *Am. J. Path.*, 1925, **1**, 1–9.
17. Cowdry, E. V. A comparison of the intranuclear inclusions produced by the herpetic virus and by virus III in rabbits. *Arch. Path.*, 1930, **10**, 23–37.
18. Pinkerton, H., and Hass, G. M. Spotted Fever. I. Intranuclear Rickettsiae in spotted fever studied in tissue culture. *J. Exper. Med.*, 1932, **56**, 151–156.
19. Wolbach, S. B. Studies on Rocky Mountain spotted fever. *J. Med. Res.*, 1919, **41**, 1–197.
20. Nicholson, F. M. A cytological study of the nature of Rickettsia in Rocky Mountain spotted fever. *J. Exper. Med.*, 1923, **37**, 221–230.
21. Cowdry, E. V. Studies on the etiology of heartwater. *J. Exper. Med.*, 1925, **42**, 231–252; 253–274.
22. Lillie, R. D. Psittacosis: Rickettsia-like inclusions in man and in experimental animals. *Pub. Health Rep.*, 1930, **45**, 773–778.
23. Goodpasture, E. W. The pathology of certain virus diseases. *South. M. J.*, 1928, **21**, 535–539.
24. Woodruff, C. E., and Goodpasture, E. W. The infectivity of isolated inclusion bodies of fowl-pox. *Am. J. Path.*, 1929, **5**, 1–9.

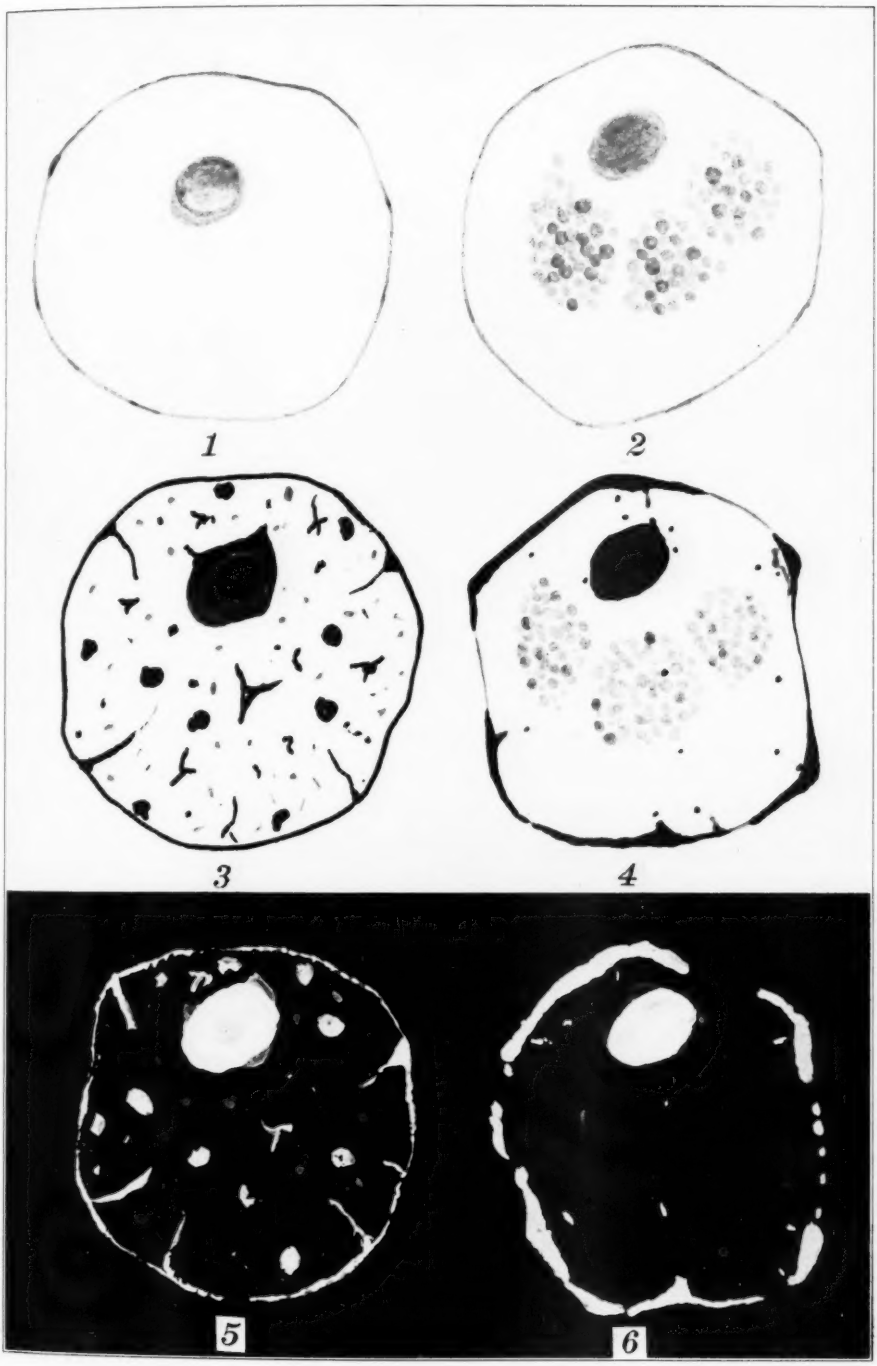
DESCRIPTION OF PLATES

PLATE 26

Diagrams made of the nuclei of liver cells of *Macacus rhesus* at a magnification of about 6000 diameters.

FIGS. 1, 3 and 5 represent a normal nucleus of a still living cell as seen mounted in physiological salt solution at high magnification with direct illumination; a similar nucleus after fixation in absolute alcohol plus 10 per cent of neutral commercial formalin and coloration by Giemsa's stain; and again a similar nucleus incinerated following the fixation just mentioned and examined in the dark-field.

FIGS. 2, 4 and 6 show nuclei containing inclusions developed under the influence of the yellow fever virus as observed under parallel conditions, using the same techniques.



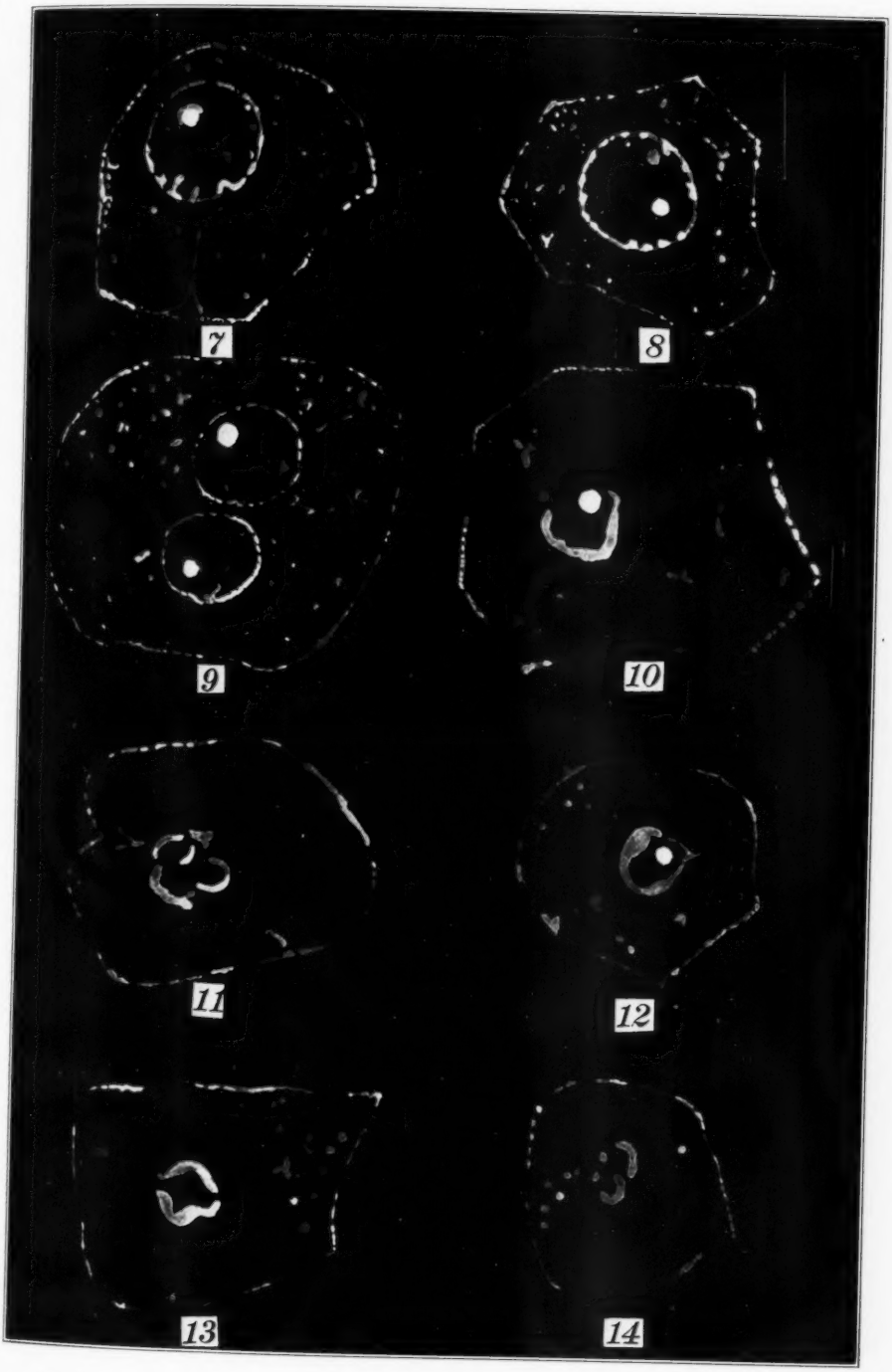
Cowdry

Microincineration of Intranuclear Inclusions

PLATE 27

Drawings of selected liver cells, viewed in the dark-field, of a *Macacus rhesus* experimentally infected with yellow fever. They were made with 3 mm. objective X, compensating ocular 20 and camera lucida at the level of the table, giving a magnification of about 1500 diameters.

FIGS. 7–9 show the cellular ash of early stages in the reaction, and FIGS. 10–14 of later and terminal stages. The distribution of the ash clearly indicates the position occupied by the cell membrane, nuclear membrane, nucleolus and other parts of the injured cells.



Cowdry

Microincineration of Intranuclear Inclusions

STUDIES ON THE PATHOGENESIS OF ERYTHROLEUCOSIS *

HERBERT L. RATCLIFFE, SC.D., AND JACOB FURTH, M.D.
WITH THE ASSISTANCE OF CHARLES BREEDIS

(From the Henry Phipps Institute and the McManes Laboratory of Pathology of the University of Pennsylvania, Philadelphia, Pa.)

Erythroleucosis, a transmissible disease of the domestic fowl resembling leucemia, was discovered by Ellermann.[1] It is characterized by large numbers of basophile (lymphoid) erythroblasts in the peripheral blood, by the multiplication of these cells in the bone marrow without maturation, and by their accumulation in large numbers in the pulp of the spleen and in the capillaries of many organs such as the liver, lungs and kidneys.

Ellermann [2] considered this disease analogous to pernicious anemia of man. He was able to transmit it to other fowls by injections of blood, organ suspensions and cell-free filtrates of blood.

Recently most of Ellermann's experiments have been amply confirmed,[3, 4] but studies in the Henry Phipps Institute have shown that erythroleucosis has the characteristics of a neoplastic process rather than of pernicious anemia.[3] It is usually accompanied by severe anemia, but it often begins with the appearance of primitive erythroblasts in the circulation at a time when signs of anemia are wanting.[3] Moreover, death may occur while there is still an ample number of red cells in the circulating blood.

The investigations referred to [4, 5] have also shown that only two types of leucosis of fowls, erythroleucosis and myeloid leucemia, are caused by the same agent, a view confirmed by the studies of Engelbreth-Holm.[6] A single animal may develop simultaneously both types of leucosis and either one may change into the other. The factors determining the types are not yet understood.

Heretofore only fully developed cases of erythroleucosis have been described. In these the changes in the bone marrow and viscera have been so advanced that the manner of their development could not be determined. The experiments reported here were undertaken to obtain information on the pathogenesis of the disease.

* This investigation has been supported by a Fund for the Study of Leucemia and Related Diseases.

Received for publication August 1, 1932.

Plan of Experiments and Technique

The pathogenesis of erythroleucosis in fowls injected with cell-free filtrates of leucemic blood or organ suspensions is not necessarily the same as that caused by injections of material containing leucemic cells. With the former inoculum some blood-forming elements of the inoculated fowl must undergo neoplastic transformation, but with the second the disease may also result from the unrestricted growth of transferred cells.[7] One group of fowls was therefore inoculated with whole blood, a second with washed blood cells, and a third with filtered plasma. These experiments were performed at a time when most of the fowls successfully inoculated developed erythroleucosis; during the earlier studies myeloid leucosis was common among fowls inoculated with this transmissible strain.

Blood smears, and in one series blood counts also, were made before the inoculation of each fowl, at 4 day intervals after inoculation, and again shortly before death (Tables I to III). The methods of blood examination employed have been described.[3, 4] The birds were killed at regular intervals by ether; those living longer than 31 days were kept under observation for varying periods of time. Fowls were autopsied and tissue for microscopic examination was taken within 1 hour after death to determine the sequence of changes that precede the well known manifestations of leucosis. Tissues were fixed in Zenker-formol solution, embedded in paraffin and stained by hematoxylin and eosin-azur II, Heidenhain's modification of Mallory's anilin blue stain, and some by a combination of Weigert's elastic tissue stain with hematoxylin and eosin-azur II. The last method is useful in attempting to trace the endothelial lining of the capillaries of the bone marrow.

Since all but two fowls, one in the first and one in the third group, that lived longer than 15 days after inoculation developed leucosis, it may be supposed that the majority of those killed before the blood gave evidence of erythroleucosis would have developed the disease had they been permitted to live.

Two fowls in the first and two in the second group recovered from leucosis, as indicated by the study of blood smears. These, with eight other fowls surviving leucosis of varying intensities, were killed at various intervals after apparent recovery for observation of tissue changes with recovery. Baumgarten,[8] it may be recalled, has assumed that in leucemia of man healing may be associated with osteosclerosis. It has also been stated that osteosclerosis accompanies leucosis of chickens.[9] The frequent occurrence of bony growth

of unknown etiology obliterating the marrow cavity of fowls adds further interest to these examinations.

In the present study attention was directed to the possible origin of the basophile erythroblasts whose intravascular multiplication characterizes erythroleucosis, and to the changes accompanying erythroblastic hyperplasia of the marrow. It may be recalled that the causative agent of leucosis of fowls is, according to Ellermann and later investigators, a filterable virus. It is maintained (McGowan [9]) nevertheless that some infections, particularly those of the respiratory tract, may extend to the marrow cavity by way of the air sacs and cause changes resembling leucosis. None of the fowls studied here had any infection of the respiratory tract.

The terms used in describing the cells of the erythrocyte series are, in the inverse order of the cellular development: erythrocyte, polychrome erythrocyte, polychrome erythroblast, basophile erythroblast. In blood smears basophile erythroblasts comprise cells varying in appearance from the typical erythroblast to those resembling primitive lymphocytes, but in sections these fine differences are lost.

In blood smears containing numerous erythroblasts, primitive cells are seen resembling lymphocytes and lacking the morphological character of erythroblasts. They were described and illustrated in a previous report [3] as "lymphoid cells." Since the available evidence points to the erythroblastic nature of these cells [3] we have included them (in Table IV) among the basophile erythroblasts.

Observations

In Tables I to IV only conspicuous deviations from the normal, as determined by comparison with organs of healthy fowls of corresponding ages, are mentioned. In summarizing the results of the blood examination the term anemia has been used when many polychrome erythrocytes and erythroblasts, and but few or no basophile erythroblasts, were seen in smears. The presence of numerous basophile erythroblasts is taken to indicate erythroleucosis. These designations are arbitrary. In the tables maturation of the red cell series is referred to when the term *maturation* is used, and by *stasis* is meant accumulation of basophile erythroblasts in the capillaries.

Table I summarizes observations on thirteen fowls (Group I), each of which was injected intravenously with 0.6 cc. of heparinized whole blood from a fowl with severe erythroleucosis.

Table II summarizes observations of ten fowls (Group II), each of which was injected intravenously with 0.6 cc. of washed blood cells from a fowl with severe erythroleucosis.

The cells were prepared as follows: Heparinized blood was centrifugalized at low speed, the plasma discarded and the cells resuspended in Locke's solution, spun again, and again suspended in a sufficient quantity of Locke's solution to equal the original volume. The cells were kept chilled throughout the process.

TABLE I

Changes in Organs of Fowls Inoculated with Whole Leucemic Blood

No. of fowl	Weight				Autopsy	Blood at last examination	Microscopic Examination		
	at inoculation	postmortem					Bone marrow	Liver	Spleen
		body	liver	spleen					
	gm.	*gm.*	*gm.*	*gm.*	*days after inoculation*				
1656	660	..	..	..	4	Negative	Normal; few erythroblasts in sinusoids with active erythrogenesis	Negative	Negative
1657	620	..	..	..	4	Negative	Normal; few erythroblasts in sinusoids with active erythrogenesis	Negative	Negative
1658	740	..	39.3	1.7	8	Anemia	Congested hyperplastic; normal maturation except in foci of basophile erythroblasts	Negative	Negative
1659	710	..	39.5	3.7	8	Negative	Normal except in a few capillaries containing numerous erythroblasts	Negative	Negative
1664	600	..	..	..	13*	Anemia	Many sinusoids filled with basophile erythroblasts; otherwise normal	Negative	Negative
1660	620	765	36.0	4.0	15	Incipient erythroleucosis	Slight hyperplasia; basophile erythroblasts numerous in many sinusoids; normal maturation except in these; slight intercapillary fibrosis	Slight stasis	Slight stasis

1661	710	900	38.0	2.5	15	Negative	Normal except a few sinusoids which contain numerous erythroblasts	Negative	Negative
1663	730	790	110.0	11.1	22	Severe erythro-leucosis	Erythroblasts fill all sinusoids; few groups of myelocytes	Pronounced stasis	Pronounced stasis
1666	610	870	35.7	3.8	22	Incipient erythro-leucosis	Erythroblasts fill all sinusoids; no maturation	Slight stasis	Slight stasis
1662	630	1030	46.3	2.0	31	Negative	Congestion and hyperplasia of red and white cell-forming tissues; normal maturation	Negative	Negative
1665	605	930	69.5	7.8	31	Erythroleucosis	Erythroblasts fill entire capillary bed; no maturation	Stasis	Stasis
1667	550	1180	47.4	3.5	86	Negative	Congestion; marked hyperplasia of all elements; normal maturation except in several large foci which are filled with erythroblasts	Negative	Perivascular necrosis; myeloid hyperplasia
1669	620	860	40.0	3.0	183	Negative	See Table V (recovered from leucosis)	Negative	See Table V

* Died.

TABLE II

Changes in Organs of Fowls Inoculated with Washed Leucemic Cells

No. of fowl	Weight				Autopsy	Blood at last examination	Microscopic examination		
	at inoculation	postmortem					Bone marrow	Liver	Spleen
		body	liver	spleen					
	gm.	*gm.*	*gm.*	*gm.*	*days after inoculation*				
1843	705	750	34.0	2.2	4	Incipient erythroleuosis	Congested, marked erythroblastic hyperplasia; maturation retarded	Negative	Negative
1844	745	750	31.0	2.3	4	Negative	Slight hyperplasia; congested; erythroblasts numerous in foci; normal maturation except in these foci	Negative	Negative
1841	765	880	38.0	1.9	8	Negative	Slight hyperplasia; few erythroblasts in sinusoids with active erythrogenesis	Negative	Negative
1842	785	850	36.0	7.0	8	Incipient erythroleucosis	Moderate intercapillary fibrosis; erythroblasts fill patent sinusoids; no maturation	Slight stasis	Marked stasis
1846	935	1060	35.0	3.0	15	Incipient erythroleucosis	Hyperplasia; congested; maturation except in many sinusoids where erythroblasts are increased	Negative	Slight stasis
1848	800	960	36.0	7.2	15	Erythroleucosis	Diffuse erythroblastic hyperplasia; no maturation; slight intercapillary fibrosis	Slight stasis	Marked stasis
1849	755	755	65.0	7.5	20*	Erythroleucosis	Diffuse erythroblastic hyperplasia; no maturation.	Stasis; tumor formation	Stasis
1850	635	900	42.0	3.0	29*	Erythroleucosis	Diffuse erythroblastic hyperplasia; no maturation	Stasis	Stasis
1847	750	1135	40.0	2.1	120	Negative	See Table V (recovered from leucosis)	Negative	Negative
1851	880	1600	51.7	6.0	120	Negative	See Table V (recovered from leucosis)	Negative	Negative

* Died.

TABLE III

Changes in Organs of Fowls Inoculated with Filtered Leucemic Plasma

No. of fowl	Weight				Autopsy	Blood at last examination	Microscopic Examination		
	at inoculation	postmortem					Bone marrow	Liver	Spleen
		body	liver	spleen					
	gm.	*gm.*	*gm.*	*gm.*	*days after inoculation*				
1902	720	780	35.0	2.6	4	Negative	Congested; basophile erythroblasts numerous in many sinusoids	Negative	Negative
1903	700	745	32.0	2.0	4	Negative	Normal; sinusoids engorged	Negative	Negative
1904	700	815	28.0	2.0	8	Negative	Normal; erythroblasts numerous in engorged sinusoids	Negative	Negative
1905	680	800	27.0	2.7	8	Negative	Normal; erythroblasts numerous in engorged sinusoids	Negative	Negative
1906	600	695	25.0	1.2	8	Negative	Erythroblasts numerous in engorged sinusoids; maturation retarded	Negative	Negative
1907	650	830	31.0	1.3	15	Negative	Slight focal erythroblastic hyperplasia; maturation reduced	Negative	Negative
1908	710	890	32.0	1.3	15	Negative	Normal	Negative	Negative
1909	720	940	33.5	1.0	15	Negative	Slight erythroblastic hyperplasia	Negative	Negative
1916	730	700	41.7	2.7	24*	Erythroleucosis	Diffuse erythroblastic hyperplasia; no maturation	Stasis	Stasis
1913	720	1070	54.6	9.6	31	Erythroleucosis	Diffuse erythroblastic hyperplasia; no maturation	Stasis	Stasis
1915	720	1200	51.0	5.1	31	Incipient erythroleucosis	Diffuse erythroblastic hyperplasia; no maturation	Stasis	Stasis
1910	770	750	85.0	20.0	46*	Erythroleucosis	Diffuse erythroblastic hyperplasia; no maturation	Stasis	Stasis
1911	600	1830	56.2	3.2	93	Negative	Normal	Negative	Negative

* Died.

Table III summarizes the record of thirteen fowls (Group III), each of which was given an intravenous injection of 3 cc. of a Berkefeld V filtrate of the plasma of a fowl with severe myeloid leucemia.

The material used for inoculation was prepared as follows: Heparinized whole blood was chilled, and then spun at high speed (1000 R.P.M.) for 10 minutes, the plasma decanted and again spun at high speed (2000 R.P.M.) for 10 minutes, and the plasma then filtered through a coarse (V) Berkefeld filter.

Leucemic blood cells and whole blood caused leucosis sooner than filtered plasma. The character of the primary change, however, appeared to be without relation to the type of the inoculum. Accordingly typical cases from each of the three groups may be combined in describing the earlier changes.

Early Organic Changes: In healthy young fowls (3 to 4 months of age) blood formation was observed throughout the midfemoral marrow; it was particularly conspicuous in the cortical region. Erythrogenesis seemed to occur in capillaries throughout the marrow. Basophile erythroblasts were very scanty, polychrome erythroblasts and polychrome erythrocytes comprising the greater number of immature cells.

The earliest lesion in erythroleucosis consisted of an increase in the number of basophile erythroblasts in scattered sinusoidal capillaries (Fig. 1). These cells are large and spherical or polygonal in shape with relatively scanty basophilic cytoplasm distributed regularly about large vesicular hyperchromatic nuclei (Figs. 3 and 4). Changes of this nature were seen in marrows of fowls Nos. 1904 and 1905 in 8 days, and in fowl No. 1909 in 15 days, after inoculation of the cell-free agent.

Following this beginning hyperplasia of basophile erythroblasts, the other immature cells of the erythrocyte series disappeared from the involved areas. There was a single or double row of basophile erythroblasts arranged along the endothelial wall of a number of sinusoidal capillaries with only mature erythrocytes in the lumen (Figs. 1 and 3). The bone marrow of fowls Nos. 1656, 1661 and 1846 illustrate this stage of the disease. Occasionally erythrogenesis both with and without maturation was seen in a single capillary cut longitudinally.

In two fowls, Nos. 1844 and 1907, there were occasional small groups of sinusoidal capillaries filled completely with basophile erythroblasts. These foci (Fig. 2) were sharply outlined and were

surrounded on all sides by apparently normal erythrocyte-forming centers.

In fowls inoculated with cell-free filtrate of leucemic blood the process seemed to continue in the marrow until most or all of the sinusoidal capillaries were involved. More capillaries became patent, fat was correspondingly reduced, and the growing basophile erythroblasts extended into the newly opened vessels. These cells were not seen to arise from endothelium or other "fixed" cells, having no continuity with the latter. The entire capillary bed of the marrow was apparently invaded before the erythroblasts appeared in the blood stream and became concentrated in the capillaries of other organs.

Fowl No. 1906 illustrates the disease moderately advanced. In the bone marrow fat was reduced to about half and the number of open capillaries correspondingly increased. Most of the capillaries contained basophile erythroblasts, but other immature cells of the erythrocyte series were absent. The erythroblasts were not seen in blood smears taken just before death or in sections of the liver and spleen.

Fowl No. 1915 illustrates the stage in the disease when the blood and viscera were being invaded by the basophile erythroblasts. The vascular bed of the marrow in this case was completely filled by these cells.

When fowls were inoculated with whole blood or blood cells basophile erythroblasts appeared in the circulating blood before the marrow was involved to any considerable extent (Tables I and II). This may be illustrated by the following cases.

Marrow from fowl No. 1843, autopsied 4 days after inoculation, contained abundant fat except at the periphery, but there were numerous basophile erythroblasts in practically all open capillaries and these cells were also seen in blood smears. The viscera were not invaded.

The lesions in fowl No. 1846 15 days after inoculation were slightly more advanced, and the marrow contained much less fat. Erythroblasts were numerous in about half of the patent sinusoids of the marrow, but the involvement was not so extensive as in fowl No. 1906 (Fig. 3). Yet there were many basophile erythroblasts in the circulating blood and in the pulp of the spleen, but the liver was free. Fowls Nos. 1666 and 1660 are further examples of the same stage. The marrow in the former contained erythroblasts in all open capillaries. These cells were also seen in blood smears, but not in the liver and spleen. In the marrow of fowl No. 1660 there were numerous erythroblasts in many open capillaries, but they were probably not so abundant as in fowl No. 1666. Yet these cells were present in the circulating blood and there was beginning stasis in the liver and spleen.

Effects Upon Other Elements of the Bone Marrow: During the earlier stages of the erythroblastic hyperplasia the myelogenic tis-

sues of the bone marrow seemed normal. In the lymphatic tissues, however, there was in two of the early cases, fowls Nos. 1659 and 1906, accumulation of large numbers of phagocytic cells containing cellular débris.

In fully developed cases of the disease no lymphatic tissue could be seen in the marrow and granulocyte formation was slight.

Hyperplasia of the intercapillary fibrous tissue was observed in three fowls. It was pronounced in one fowl (No. 1842) and mild in two (Nos. 1660 and 1848). The capillaries throughout the marrow of fowl No. 1842 were separated by extensive proliferation of fibrous tissue. Fibrosis was most conspicuous at the periphery of the marrow where it caused pronounced reduction in the vascular bed (Fig. 5). Ossification was not observed in these areas.

Changes in the Blood and Viscera: It has been seen that in fowls inoculated with cell-free material, erythroblasts did not appear in the circulation until the bone marrow was filled with them, but that in fowls inoculated with whole blood and washed cells the erythroblasts may be present in the circulating blood and in the capillaries of the liver and pulp of the spleen before the bone marrow is involved to any considerable extent (see fowls Nos. 1843, 1846, 1666 and 1660). It was previously shown,[7] however, that when injected into the blood stream of susceptible hosts the leucemic cells of the fowl are capable of multiplication outside the marrow.

Stasis of erythroblasts in the capillaries of certain organs, notably of the lungs, liver, spleen and kidneys, seems to begin soon after these cells appear in the circulating blood. The liver, spleen and lungs are the first to be involved. Stasis in other organs is less conspicuous.

In the livers of fowls Nos. 1660 and 1842, incipient cases, erythroblasts were seen clumped together or singly in numerous capillaries. These are examples of beginning stasis. Mitoses were frequently observed among these cells, indicating multiplication in this location.

In fully developed erythroleucosis stasis of erythroblasts apparently may cause much damage to the liver, *e.g.* fowls Nos. 1849 and 1850. In the former the accumulation of the erythroblasts in the liver capillaries amounted to small tumors, in many sites expanding the capillary walls and compressing the liver cells. This was accompanied by the formation of thrombi in the portal vein. In fowl No. 1850 parts of the liver lobules were undergoing necrosis.

Damage to the spleen was seen in two fowls (Nos. 1667 and 1669) that had recovered from erythroleucosis. This consisted of bands of hyaline material about the arteries.

The factors causing stasis of these cells in some capillaries, *e.g.* in liver, spleen and lungs, but not in others, *e.g.* in voluntary muscle, are not understood. It is noteworthy that stasis is localized in the capillaries known to be active in the removal of foreign particles introduced into the circulation.

Absence of Specific Inflammatory Changes: McGowan [9] states that leucosis of fowls is the response of the marrow to acute infection. Recently Cash and Doan [10] concluded that in pigeons infection by *Bacillus aertrycke* may simulate leucemia. This similarity consists of extreme myeloid hyperplasia associated with an increase of the circulating leucocytes. White blood counts in leucemia are, however, much higher than in *B. aertrycke* infection and the leucocytes in leucosis are of the more immature types. The relation of leucocytosis to myeloid leucosis of the fowl will be discussed in a later report.

None of the fowls studied has shown signs suggestive of acute infection.

The Origin of the Basophile Erythroblasts: As to the source of basophile erythroblasts in erythroleucosis three possibilities were considered: (*a*) erythroblasts of the host which the transmissible agent has entered, (*b*) inoculated basophile erythroblasts, and (*c*) the endothelial cells of the sinusoids of the marrow.

In fowls injected with whole blood or washed cells, the basophile erythroblasts rapidly multiplying in the marrow may be derived from those injected. In fowls injected with cell-free material, however, the basophile erythroblasts must arise from preëxisting cells of the marrow stimulated to growth by the filterable transmitting agent.

Since there was no definite evidence of endothelial hyperplasia in the intersinusoidal capillaries of the marrow, such as has been described in birds made anemic by starvation,[11] nor in the sinusoidal capillaries where hyperplasia of the basophile erythroblasts is first seen in erythroleucosis, it may be assumed that the majority of these cells are derived from similar basophile erythroblasts already present in the bone marrow capillaries. Mitoses among the erythroblasts are seen frequently, but the fixed cells have not been seen to divide.

Blood Changes Accompanying the Development of Erythroleucosis: The blood picture in advanced cases of erythroleucosis has been described and illustrated in a previous report.[3] In most instances it is that of a severe anemia characterized by relatively large numbers of basophile erythroblasts (erythroleucosis of

the erythroblastic type). In some cases, however, primitive erythroblasts resembling lymphocytes[3] are abundant in the circulation (50,000 to 300,000) whereas cells with the characteristic structure of erythroblasts and polychrome erythrocytes are few (erythroleucosis of the primitive cell type).

The appearance of the blood smear in each fowl studied here is stated in Tables I to III. More detailed illustrations of the early blood changes are given in Table IV.

No. 1843: Four days after inoculation there was no reduction in the number of red blood cells though the presence of immature red cells suggested incipient erythroleucosis. Note furthermore an increase in the number of thrombocytes. The bone marrow of this fowl showed in the cortical areas increase of primitive erythroblasts, a lesion suggestive of incipient erythroleucosis.

No. 1844: The second fowl of this series, killed 4 days after inoculation, had no immature cells in the circulation. The bone marrow showed a few focal accumulations of basophile erythroblasts, apparently incipient lesions of erythroleucosis.

No. 1841: The blood smear of this fowl, killed 8 days after inoculation, showed no marked abnormalities. In the bone marrow there were no lesions suggestive of leucosis.

No. 1842: Four days after inoculation erythroblasts were already numerous in the circulation of this fowl, although there was no reduction in the number of erythrocytes. Note the pronounced increase in the number of thrombocytes 4 days after inoculation followed by a sharp drop of these cells. At *postmortem* examination, changes indicative of advanced erythroleucosis were observed in the bone marrow and there was stasis of basophile erythroblasts in the liver and spleen.

No. 1848 represents a more advanced stage of the disease. Note that there were few polymorphonuclear leucocytes and the thrombocytes were practically absent from the circulation 15 days after inoculation. There was mild stasis in both the liver and spleen.

No. 1851 is given as an example of leucosis ending in recovery. The differential counts leave no doubt as to the correctness of diagnosis. Eight days after inoculation the blood smear indicated erythroleucosis, but a week later many primitive large mononuclear cells (myeloblasts according to Ellermann) appeared in the circulation, suggesting myeloid involvement. At the height of the disease the number of leucocytes was estimated to exceed 300,000, there were mitotic figures in the circulation and there was an absence of thrombocytes, which had been preceded by a transient increase. A few myelocytes seen in the blood smear of this chicken 15, 27 and 45 days after inoculation have been included among the polymorphonuclear leucocytes in Table IV. Recovery was complete, for the bone marrow showed only slight non-specific hyperplasia of erythrogenic and myelogenic elements.

Progressive hyperplasia of erythroblasts with failure to mature is naturally followed by anemia. Evidence for a primary blood destruction is wanting. The replacement of the marrow by primitive cells interferes also with the formation of granulocytes, lymphocytes and thrombocytes. Lymphatic tissues being abundant in

TABLE IV

Blood Changes in Transmissible Leucosis

No. of fowl	Time of examination	Hemoglobin (Sahli)	Red blood count	White blood count	Polychrome erythrocytes	Polychrome erythroblasts	Basophile erythroblasts	Thrombocytes	Differential Count: lymphocytes	monocytes	polymorphonuclears	mast cells	primitive mononuclears
			in thousands		per 100 white cells								
1843	Before inoculation	47	2295	32	0	0	0	120	78	9	10	3	0
	4 days after inoculation	46	2355	28	numerous	35	4	223	66	3	29	1	1
1844	Before inoculation	49	2415	31	0	0	0	82	69	9	21	1	0
	4 days after inoculation	42	1995	40	0	0	0	105	52	17	28	3	0
1841	Before inoculation	41	2165	42	0	0	0	42	72	9	16	3	0
	4 days after inoculation	45	2470	49	1	0	0	86	75	8	12	5	0
	8 days after inoculation	49	2730	46	0	0	0	55	80	3	16	1	0
1842	Before inoculation	42	2198	35	2	0	0	80	71	6	21	2	0
	4 days after inoculation	47	2365	25	21	11	2	181	60	13	26	1	0
	8 days after inoculation	41	1735	21	26	33	13	15	86	4	10	0	0
1848	Before inoculation	48	2470	37	0	0	0	64	56	15	28	1	0
	4 days after inoculation	–	–	–	0	0	0	115	80	9	9	2	0
	8 days after inoculation	–	–	–	20	9	3	20	84	3	12	1	0
	15 days after inoculation	29	1770	25	10	9	4	0.5	88	7	4	1	0
1851	Before inoculation	51	2132	38	1	0	0	72	85	4	9	2	0
	8 days after inoculation	–	–	–	13	6	3	125	74	7	16	3	0
	15 days after inoculation	–	–	–	10	18	3	3	70	12	11	1	6
	27 days after inoculation	–	–	300	many	24	11	0	1	1	1.5	0	96.5
	42 days after inoculation	–	–	100	many	12	2	9	35.5	1	1.5	1	61
	49 days after inoculation	–	–	–	0	0	0	40	76	3	8	9	4

NOTE: – = not done; 0 = negative.

other locations there is no diminution in the number of lymphocytes, but disappearance of thrombocytes and decrease of the number of granulocytes usually accompany the disease.

It is noteworthy that in all instances of erythroleucosis there was an almost complete disappearance of thrombocytes from the circulation. This decline was preceded by a transient rise with the appearance of atypical thrombocytes in the circulation (in fowls Nos. 1843 and 1842 4 days after inoculation, in Nos. 1848 and 1851 8 days after inoculation). These thrombocytes were larger than normal, more nearly round, and contained many vacuoles. They showed great individual differences in size and form (Fig. 12).

Recovery in Erythroleucosis: In an attempt to determine the successive changes that occur during apparent recovery, fowls surviving leucosis were killed at various intervals after the disappearance of immature cells from the peripheral blood.

In Table V notes are presented on the microscopic appearances of the bone marrow, liver, and spleen, together with other data on ten fowls that had recovered from leucosis of varying intensities. When hyperplasia is mentioned both red and white cell-forming tissues were involved unless otherwise indicated.

Recovery from erythroleucosis seems to be in part a reversal of the changes occurring in its development. The disappearance of leucemic cells from the peripheral blood is apparently preceded by or simultaneous with the disappearance of these cells from the capillaries of the internal organs.

Fowl No. 1667, Table I, and fowl No. 2154, Table V, illustrate the early stage in the return to normal. The course of the disease of the first fowl is similar to that in No. 1851 described in Table IV. When autopsied there were still a few immature erythrocytes in the circulating blood, but the bird was apparently on the way to recovery. Both myelogenic and erythrogenic elements of the marrow were hyperplastic, but maturation appeared normal, except in several large foci made up of sinusoids filled only with erythroblasts (Fig. 6). The capillaries of the liver were free of erythroblasts and granulocytes seemed to be forming in the pulp of the spleen. The microscopic appearances were similar in fowl No. 2154 whose blood seemed normal before death. The liver and spleen were free of lesions. The marrow was hyperplastic, but maturation appeared normal except in occasional foci of sinusoids filled with erythroblasts.

More advanced regressive changes were seen in fowls Nos. 1924 and 1994. In the former the blood was negative for 17 days before autopsy. The liver and spleen were found to be free of lesions. The bone marrow was hyperplastic, but maturation of red and white cells seemed normal. In one small area the marrow was being invaded by a solid growth of large spindle cells of regular shape with

TABLE V

Notes on Fowls Recovering from Leucosis

No. of fowl	Duration of leucosis *	Time since recovery	Microscopic Changes		
			Bone marrow	Liver	Spleen
	days	*days*			
1048	31	417	Marrow cavity partly obliterated by spongy bone; marrow hyperplastic	Negative	Negative
1669	48	118	Marrow cavity partly obliterated by bone; marrow hyperplastic	Negative	Myelogenesis in pulp, hyaline necrosis about arteries
1847	4	92	Normal erythrogenesis; very active myelogenesis	Negative	Negative
1836	42	91	Slight hyperplasia	Negative	Negative
1851	41	71	Slight hyperplasia	Negative	Negative
1923	37	47	Normal	Negative	Negative
1934	54	20	Normal	Negative	Negative
1994	24	19	Pronounced congestion and hyperplasia; tumor formation (see text)	Negative	Negative
1924	59	17	Pronounced congestion and hyperplasia; tumor formation (see text)		
2154	37	7	Pronounced congestion and hyperplasia; maturation of erythrocytes and granulocytes normal, except in a few sinusoids which contain large numbers of erythroblasts	Negative	Negative

* As indicated by blood smears. At time of death the blood smears of all these fowls seemed normal.

pale vesicular nuclei. No other tumorous growth was seen elsewhere in the body of this fowl and this small area was discovered only during microscopic examination. Its appearance (Fig. 10) suggests a neoplasm arising apparently from elements of the marrow.

The blood in fowl No. 1994 had been negative for 19 days and the liver and spleen were free of lesions. In part of the hyperplastic femoral marrow there was normal maturation of both red and white cells. In other parts there was a tumorous growth of large spindle cells with hyperchromatic pleomorphic nuclei (Fig. 9). These cells formed a coarse network enclosing numerous blood spaces. The growth apparently was expanding within the marrow and encroaching on the normal parts.

In three fowls killed 47, 71 and 91 days after inoculation, there was slight nonspecific hyperplasia of both erythrocytopoietic and granulocytopoietic elements of the marrow. In fowl No. 1847 killed 92 days after the blood became negative the myelogenic tissues were hyperplastic (Figs. 7 and 8).

In two fowls, Nos. 1048 and 1668, examined 417 and 118 days after recovery, the marrow cavity of the femur was almost completely filled by growth of spongy bone in which areas of active marrow were enclosed (Fig. 11). There was scant fibrous tissue about the trabeculae. Bony changes of this nature do not seem to be an essential part of recovery from leucosis; they are not infrequently seen in older fowls. More recently we have examined five additional fowls that recovered from transmissible leucosis of from 15 to 58 days duration. The *postmortem* examination was performed from 15 to 88 days after apparent recovery and osteodystrophy was not seen in these fowls.

On the basis of these observations recovery from erythroleucosis may be visualized as follows. The multiplication of erythroblasts is reduced or ceases, as is evidenced by the lack of mitotic figures among them. Polychrome erythroblasts become numerous and are seen in division. Polychrome erythrocytes and mature erythrocytes are present in large numbers in the sinusoids so that the marrow has the appearance seen with anemia. Whether the basophile erythroblasts mature or are replaced by erythroblasts capable of maturation cannot be determined by examination of fixed material.

Discussion

Blood-Forming Systems Affected: The observations previously reported [4, 5, 6] support the view that the causative agent of transmissible leucosis of fowls may stimulate either the erythrogenic or myelogenic tissues of the bone marrow, but not the lymphatic tissues. In the series described here there was little evidence of associated myeloblastic hyperplasia, a condition not uncommon among other fowls inoculated with this transmissible strain. Thus it is shown that the agent of transmissible leucosis is selective in its action under conditions that are, however, not yet understood.

Battaglia and Leinati [12] deny the existence of lymphatic leucosis in the fowl and state that the agent transmitting leucosis of fowls may cause a primitive cell hyperplasia resulting in erythroleucosis, myeloid leucosis, or "hemocytoblastic myelosis," but the latter type is apparently identical with lymphatic leucosis of Ellermann and other investigators.

Much of the confusion of the literature on the subject of leucosis of fowls may be traced to the large number of terms proposed to designate apparently identical cells and types of disease. Ellermann seems to have described adequately all common types of leucosis. An acceptance as far as possible of his terminology is therefore desirable. The correctness of his views on erythroleucosis and the causation of erythroleucosis and myeloid leucemia by the same filterable agent are now amply confirmed. The pathogenesis of myeloid and lymphatic leucemia of Ellermann, however, requires further investigations.

Origin of Basophile Erythroblasts: The views as to the origin of erythroblasts have recently been compiled by Michels.[13] It appears from our material that in both secondary anemia and erythroleucosis the basophile erythroblasts increase in number. The increase is moderate in experimental anemia and is accompanied by maturation of erythroblasts; it is extensive in erythroleucosis, with little or no maturation. Neither hyperplasia nor hypertrophy of endothelium is evident in these conditions.

These studies point to the basophile erythroblast as the "stem cell" of the erythrocyte series in the adult fowl. These cells are, according to our observations, capable of rapid multiplication under the influence of the agent of transmissible leucosis as well as under the action of acetylphenylhydrazin.[3] Stimulated by the former they fail to mature and erythroleucosis results; by the latter there is active maturation of erythrocytes. Whether there is also occasional formation of basophile erythroblasts from fixed cells such as endothelium remains to be proved (Stockard [14]).

The neoplastic character of leucemic lymphocytes of mice has been demonstrated by recent transmission experiments.[15] In the fowl it has been shown by transfusion experiments with blood from fowls with myeloid leucemia [7] that the large primitive mononuclear cells (myeloblasts, Ellermann) are capable of autonomous growth in susceptible hosts. Basophile erythroblasts apparently have the same property. Autonomous growth of the injected cells is taken to explain the rapid development of leucosis in fowls injected with cells, as compared with fowls inoculated with filtered plasma. This property also is the probable explanation of the involvement of organs before complete overgrowth of these cells in the marrow.

Effect on Thrombocyte Formation: Some of the observations re-

ported here bear on the origin of the thrombocytes. Concerning the conflicting views on the origin of the thrombocytes of the fowl see Michels,[13] and Jordan and Speidel.[16] The uncertainty of our knowledge of the origin of the thrombocyte of birds is emphasized by Forkner [17] who states his failure to find these cells in the marrow. Yet our studies suggest that the thrombocytes originate in the marrow, or from elements of the marrow. A drop in the number of thrombocytes preceded by a transient rise is one of the earliest changes observed in the blood with erythroleucosis. Severe erythroleucosis is often associated with an almost complete absence of thrombocytes, preceded by the appearance of atypical forms, whether young or pathological cells. Since the bone marrow is the only organ whose function is impaired in the earliest stage of transmissible leucosis it seems probable that the formation of thrombocytes is traceable to this organ. The earliest lesions commonly affect erythrogenesis and the rapid decrease of the number of circulating thrombocytes may be due to a linkage of thrombogenesis to erythrogenesis.

Other explanations, however, may be advanced. (*a*) The agent may have a direct destructive action on the thrombocytes. (*b*) Erythroblastic hyperplasia may replace the thrombocyte-forming element. The rapidity of the disappearance of these cells from the circulation also implies that the thrombocytes have a shorter life than the red cells. (*c*) In erythroleucosis the thrombocytes may be prevented from entering the circulation.

The observations of Jordan and Speidel [16] are noteworthy in connection with our findings. In the Salamander, *Triturus viridescens*, the spleen is the main organ for both erythrocytopoiesis and thrombocytopoiesis, the liver for granulocytopoiesis. After splenectomy differentiation of both erythrocytes and thrombocytes takes place in the general circulation. By the presence of fine granulations, stained red by Wright's stain in the thrombocytes and their precursors, the origin of the thrombocytes, they find, can be traced to hemoblasts (hemocytoblasts) through a series of cell forms paralleling the erythrocytic series.

Recovery in Erythroleucosis: The disappearance of the enormous numbers of immature cells from the circulation and from the capillaries of the viscera and bone marrow has some analogy with regression and absorption of transplanted tumors. It is possible, however, that in recovery from leucosis the leucemic erythroblasts, unlike tumor cells, resume normal maturation. That leucemic cells may mature rapidly under favorable conditions is suggested by the tissue culture studies of Timofejewsky and Benewolenskaja [18] which, however, have not as yet been confirmed.

Hyperplasia of intercapillary fibrous tissue resulting in fibrosis in some fowls and tumor formation in others was perhaps caused, directly or indirectly, by the leucemic agent. Osteodystrophia (osteitis) obstructing the marrow cavity, observed in two of fifteen fowls recovering from leucosis, was perhaps also connected with the disturbed function caused by the leucemic agent. The evidence is inconclusive, however, because of the insufficient number of control fowls (fowls not infected with the leucemic agent). Osteodystrophia was also seen among fowls not inoculated with leucemic material; on the other hand it accompanied almost all instances of severe chronic (spontaneous) anemia.

Conclusions

Under the influence of a filterable agent, the basophile erythroblasts of the sinusoidal capillaries of the marrow undergo unrestricted multiplication. The erythroblasts thus formed fail to mature. They crowd out all other elements of the marrow, secondarily invade the circulation, and accumulate in the capillaries of internal organs where they continue multiplication.

Fowls inoculated with material containing erythroleucotic cells showed growth of these cells in the blood stream and organs at a time when erythroblasts had only partly filled the capillary bed of the marrow. In fowls injected with the cell-free material the blood did not contain these immature cells until the marrow was almost completely filled by them.

With erythroleucosis thrombocytes in the blood stream are at first increased and later much diminished or absent. With disturbance of erythrogenesis formation of thrombocytes is inhibited.

REFERENCES

1. Ellermann, V. The Leucosis of Fowls and Leucemia Problems. Gyldendal, London, 1922.
2. Ellermann, V. Untersuchungen über die Histologie der perniziösen Anämie. *Virchows Arch. f. path. Anat.*, 1920, **228**, 247–275.
3. Furth, J. Erythroleukosis and the anemias of the fowl. *Arch. Path.*, 1931, **12**, 1–30.
4. Furth, J. Observations with new transmissible strain of leucosis (leucemia) of fowls. *J. Exper. Med.*, 1931, **53**, 243–267.
5. Stubbs, E. L., and Furth, J. Transmission experiments with leucosis of fowls. *J. Exper. Med.*, 1931, **53**, 269–276.
6. Engelbreth-Holm, J. Bericht über einen neuen Stamm Hühnerleukose. *Ztschr. f. Immunitäts forsch. u. exper. Therap.*, 1931, **73**, 126–136.

7. Crank, R. P., and Furth, J. Fate of leucemic blood of fowls after transfusion. *Proc. Soc. Exper. Biol. & Med.*, 1931, **28**, 987–989, and *Arch. Path.*, 1932, **14**, 660.
8. Baumgarten, P. Quoted by Askanazy, M., in Handbuch der pathologischen Anatomie und Histologie, Henke, F., and Lubarsch, O. J. Springer, Berlin, 1926, **1** Pt. 2, 891.
9. McGowan, J. P. Pernicious Anaemia, Leucaemia and Aplastic Anemia. Paul Hoeber, Inc., New York, 1927.
10. Cash, J. R., and Doan, C. A. Spontaneous and experimental infection of pigeons with B. aertrycke. *Am. J. Path.*, 1931, **7**, 373–398.
11. Sabin, F. R. Bone marrow. *Physiol. Rev.*, 1928, **8**, 191–244.
12. Battaglia, F., and Leinati, L. Malattie sistemiche transmissibili degli organi emopoietici del pollo con ricerche sugli elementi morfologici del sangue normale e loro genesi. *Boll. d. Ist. sieroterap. milanese*, 1929, **8**, 9.
13. Michels, N. A. Erythropoiesis. Critical review of literature. *Folia hæmat.*, 1931, **45**, 75–128.
The erythrocyte. A critical review of its normal and pathologic morphology and physiology with data on the normal red cell count and technic. *Haematologica, II Recen.*, 1931, **2**, 101–158.
14. Stockard, C. R. The origin of blood and vascular endothelium in embryos without a circulation of the blood and in the normal embryo. *Am. J. Anat.*, 1915, **18**, 227–327.
15. Seibold, H. R., Rathbone, R. R., and Furth, J. Studies on transmissible lymphadenosis of mice. *Proc. Soc. Exper. Biol. & Med.*, 1932, **29**, 629–631.
16. Jordan, H. E., and Speidel, C. C. Hemocytopoietic effect of splenectomy in the salamander, *Triturus viridescens*. *Am. J. Anat.*, 1930, **46**, 55–84.
17. Forkner, C. E. Blood and bone marrow cells of domestic fowl. *J. Exper. Med.*, 1929, **50**, 121–141.
18. Timofejewsky, A. D., and Benewolenskaja, S. W. Neue Beobachtungen an lymphoiden Zellen der myeloiden und lymphatischen Leukämie in Explantations-Versuchen. *Arch. f. exper. Zellforsch.*, 1929, **8**, 1–34.

DESCRIPTION OF PLATES

PLATE 28

Magnifications indicated are approximate.

FIG. 1. Incipient erythroleucosis: bone marrow of fowl No. 1906. Capillaries contain many basophile erythroblasts along the endothelial walls. × 200.

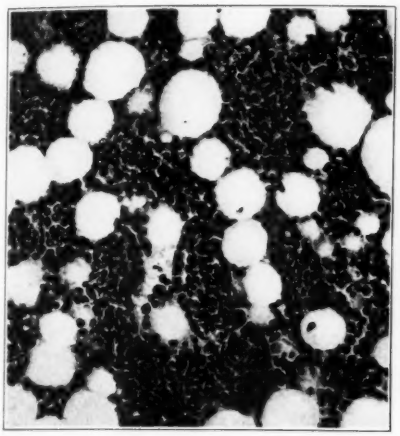
FIG. 2. Incipient erythroleucosis: bone marrow of fowl No. 1907. Focal erythroblastic hyperplasia without maturation. × 200.

FIG. 3. Incipient erythroleucosis: bone marrow of fowl No. 1906. A field similar to that seen in Fig. 1 shown at higher power. Note the lack of transitional forms between basophile erythroblasts and mature erythrocytes. × 350.

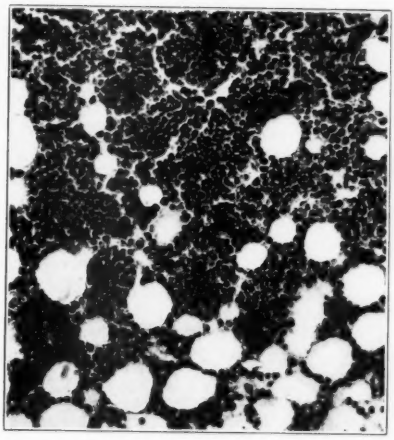
FIG. 4. Incipient erythroleucosis: bone marrow of fowl No. 1907. In this capillary cut longitudinally two mitoses are seen among the basophile erythroblasts, which almost entirely fill the capillary. × 500.

FIG. 5. Intercapillary fibrosis: bone marrow of fowl No. 1843. Capillaries contain many basophile erythroblasts. × 350.

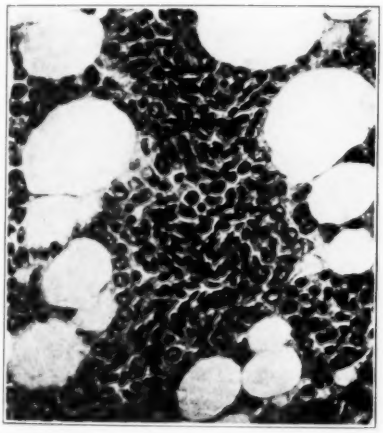
FIG. 6. Latent erythroleucosis: bone marrow of fowl No. 1667. Showing the edge of a focus of basophile erythroblasts in a fowl whose blood smears indicated recovery from erythroleucosis. × 300.



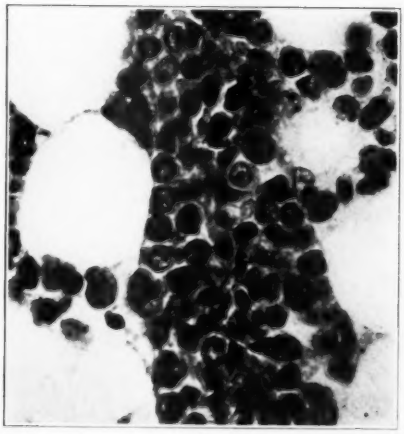
1



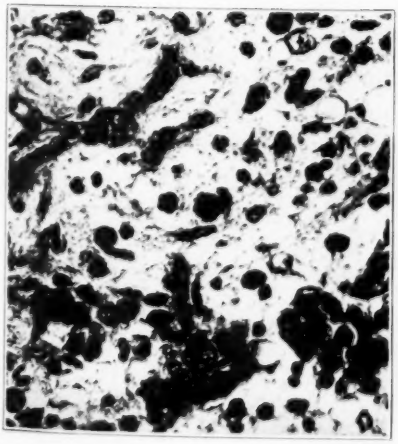
2



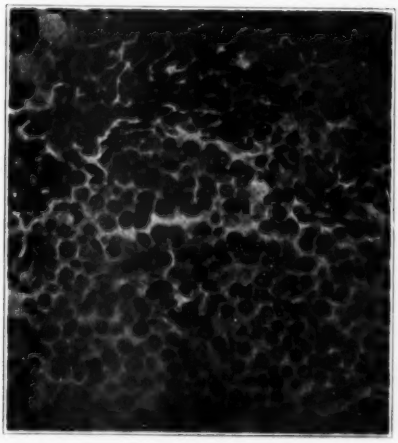
3



4



5



6

PLATE 20

FIG. 7. Recovery from erythroleucosis, suggested in blood smears: bone marrow of fowl No. 1847, showing granulocytic hyperplasia. × 150.

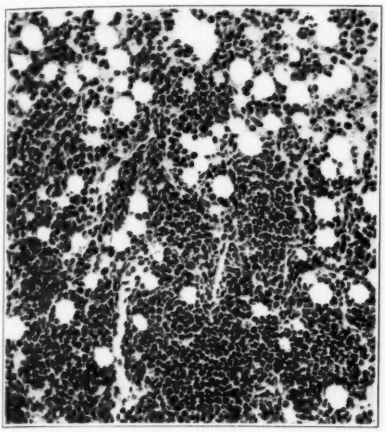
FIG. 8. Same as Fig. 7 at a higher power. × 300.

FIG. 9. Sarcomatoid growth in bone marrow of fowl No. 1094, recovering from erythroleucosis. × 300.

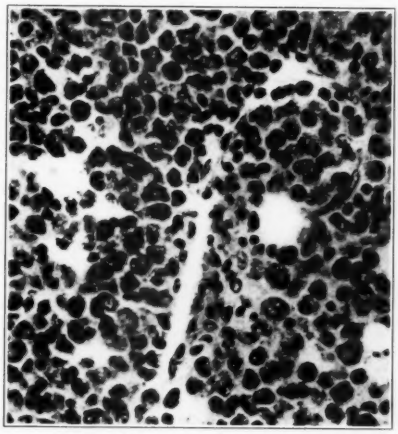
FIG. 10. Sarcomatoid growth in bone marrow of fowl No. 1924, recovering from erythroleucosis. × 300.

FIG. 11. Osteodystrophia (osteitis) fibrosa: femur of fowl No. 1047, recovering from erythroleucosis. × 20.

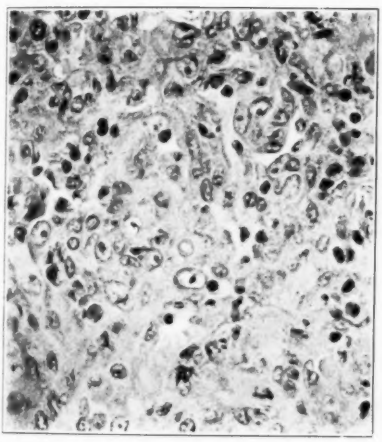
FIG. 12. Atypical thrombocytes: blood smear of fowl No. 1843, 4 days after inoculation. The central figure shows two normal thrombocytes, and the outer figure three atypical thrombocytes. × 900.



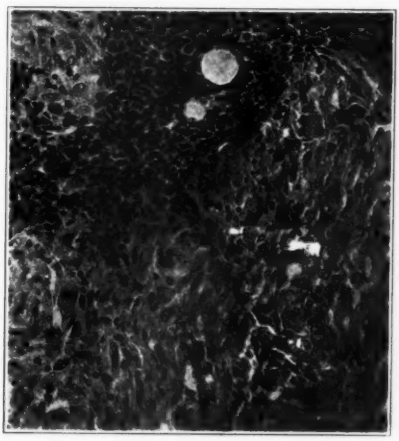
7



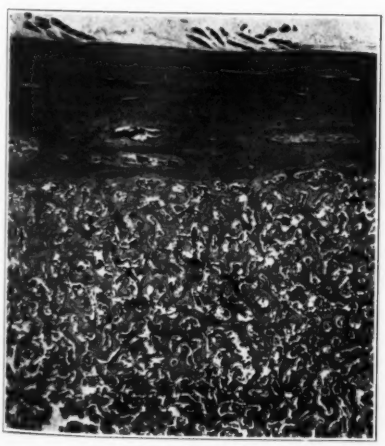
8



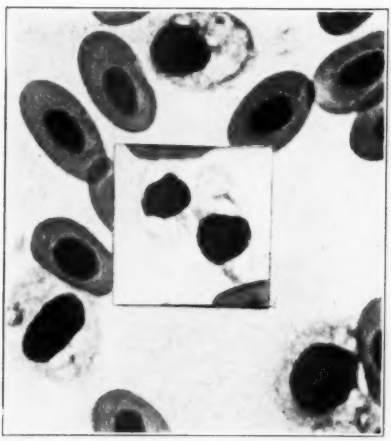
9



10



11



12

Ratcliffe, Furth and Breedis

Studies on the Pathogenesis of Erythroleucosis

AMYLOID DISEASE OF THE KIDNEYS *

E. T. BELL, M.D.

(From the Department of Pathology, University of Minnesota, Minneapolis, Minn.)

This paper is based upon a study of 65 cases of amyloid disease of the kidneys and deals chiefly with the finer structural changes in the glomeruli. Only those publications are reviewed which are concerned with structural and functional alterations in the kidneys.

It is well known that the kidneys are often free of amyloid in the presence of extensive deposits of this substance in the liver or spleen, and that in rare instances amyloid is found only in the kidneys. In Raubitschek's series [1] of 72 cases of renal amyloidosis there were 2 without amyloid in the liver or spleen. In our series of 65 cases there were 2 in which amyloid was present only in the kidneys. The circumstances that determine the site of the amyloid deposit are entirely unknown.

Forty-five cases occurred in males and 20 in females. Since the proportion of adult males to adult females is about 2 to 1 in our postmortem records, it may be concluded that sex is without influence in amyloid disease. The distribution by decades, as well as the etiological factors, is shown in Table I. Thirty-three of the 65 cases were caused by tuberculosis. In every instance the tuberculous lesions were associated with extensive chronic suppuration; there were cavities in the pulmonary tuberculous lesions, and burrowing abscesses in the instances of Pott's disease and tuberculosis of the hip.

There is general agreement that chronic suppuration is the usual cause of amyloidosis. Suppurative lesions were present in 57 of our 65 cases. In 4 instances of tertiary syphilis in our series there was chronic infection but no accumulation of pus. Amyloid has also been found with Hodgkin's disease (Bannick and Barker [2]), with multiple myelomas (Magnus-Levy,[3] Weber [4]), and with other non-suppurative diseases. Occasionally no cause of amyloidosis is found. Fahr [5]

* This investigation was supported by a grant from the Ella Sachs Plotz Foundation.
Received for publication September 1, 1932.

reported such a case in 1918, and there were 4 in our series in which no explanation of the amyloid was found.

It may be seen in Table I that tuberculosis is a much more important cause of amyloid disease before the age of 50 years (30 of 52 cases) than it is in the later decades (3 of 13 cases).

Since the disturbance of renal function is related to the amount of amyloid deposited in the kidneys, the 65 cases have been arranged in four groups: Group A, those with only a few glomerular capil-

TABLE I

Distribution of Renal Amyloidosis According to Decades and with Respect to the Associated Disease

Associated disease	1–10 years	10–20 years	20–30 years	30–40 years	40–50 years	50–60 years	60–70 years	70–80 years	Total
Tuberculosis: Lungs	..	1	3	6	10	2	..	1	23
Tuberculosis: Spine	1	3	2	2	..	..	..	..	8
Tuberculosis: Hip joint	..	2	..	..	..	..	..	..	2
Chronic abscess	..	1	1	2	2	..	..	..	6
Chronic osteomyelitis	..	1	2	1	1	..	..	..	5
Empyema	..	..	3	..	1	..	..	..	4
Pyonephrosis	..	..	..	..	1	1	..	..	2
Transverse myelitis (secondary infections)	..	2	..	..	..	..	..	..	2
Tertiary syphilis	..	..	..	1	..	1	1	1	4
Ulcerative enteritis	..	..	..	..	..	1	..	..	1
Bronchiectasis	..	..	..	..	..	1	..	..	1
Ulceration of legs	..	..	..	..	..	1	..	..	1
Unresolved pneumonia	..	..	1	..	..	..	..	..	1
Chronic arthritis	..	..	..	..	1	..	..	..	1
None	..	..	..	1	..	2	..	1	4
Total	1	10	12	13	16	9	1	3	65

laries filled with amyloid; Group B, those with moderate amyloid deposits but without complete obstruction of any glomeruli and without tubular atrophy; Group C, those with massive deposits in the glomeruli obstructing many of the capillaries and causing slight tubular atrophy; and Group D, those with extreme glomerular obstruction and marked tubular atrophy. On anatomical grounds Group C would be expected to show a moderate and Group D a marked renal insufficiency.

With the exception of the 4 instances in which no cause of amyloidosis was found, the chief clinical symptoms were those of the major illness. The appearance of albuminuria or edema first directed

attention to the kidneys. In a number of instances of tuberculosis of several years duration there was no edema or albuminuria until a few months before death.

Albuminuria: Albumin was found in all but 4 instances in which the urine was examined. The records shown in Tables II–V are those of the last urine examination. Albumin was frequently absent until a few months before death. In 2 of the cases without albumin only small amounts of amyloid were present (Group A). In the single instance in Group B (31–1208) without albuminuria, the last examination was made five months before death, and it is probable that albumin was present later on. In the case in Group D without albumin (32–52), the record is that of a single examination made one month before death. It may be concluded that the absence of albumin in the urine is almost conclusive evidence that very little or no amyloid is present in the kidneys at that time.

The presence of albuminuria, however, in a suspected case is not sufficient evidence to warrant a diagnosis of renal amyloidosis. In 20 cases of chronic suppurative diseases in which no amyloid was found in the kidneys postmortem, the urine was free from albumin in 10 instances, contained a trace in 6, and a small amount in 4. All of these 20 cases were of the type in which amyloid commonly develops, and it was found in the liver and spleen in most of them. It is well established that a chronic toxemia may injure the glomerular capillaries and cause albuminuria without the accumulation of amyloid, but our records indicate that a heavy albuminuria in a suspected case is strongly suggestive of amyloid involvement of the kidneys. A study of Groups A, B, C and D shows, however, that there is no close correlation between the degree of albuminuria and the extent of the amyloid infiltration of the kidneys.

The albumin in the urine escapes from the blood through injured glomerular capillaries. The evidence that it escapes through the glomeruli and not through tubules has been fully discussed by Ekehorn.[6] It will be explained later that there is evidence of injury of the glomerular capillaries in nearly all amyloid kidneys.

The Urinary Proteins: There are comparatively few studies available on the composition of the urinary proteins in amyloid disease. Geill [7] found very low values for the albumin fraction, 35 to 60 per cent in amyloid nephrosis, whereas in chronic nephritis and lipoid nephrosis it is usually about 90 per cent. In acute nephritis he found

that the albumin fraction was at first often lower, 65 to 70 per cent, but later it increased. In amyloid nephrosis he sometimes found that globulin exceeded albumin in the urine.

Hiller, McIntosh and Van Slyke [8] found the albumin-globulin ratio of the urine proteins usually above 10 in nephrosis, between 5 and 10 in acute nephritis, and usually below 5 in chronic nephritis with retention of urea. In 1 case of amyloid nephrosis the albumin-globulin ratio was very low, 1.5.

Lemierre and his associates [9] studied the urinary proteins in a case of amyloid nephrosis with retention of nitrogen. Three examinations of the urine showed: albumin 6.50, globulin 1.35; albumin 6.63, globulin 1.65; albumin 15.40, globulin 9.40, grams per liter of urine.

The Serum Proteins: A few observations are available on the serum proteins in renal amyloidosis. Linder, Maxwell and Green [10] found the serum proteins 3.8 to 4.7 gm. in a boy with marked retention of urea. Apparently some of the observations were made when the patient was free of edema. Bannick and Barker [2] studied the serum proteins in a patient with moderate renal insufficiency but without edema. The total protein was 3.8 gm. of which 27 per cent was albumin. Lemierre and his colleagues [9] made three observations on the serum proteins on a patient who had renal insufficiency, but was not edematous: albumin 3.26, globulin 3.22; albumin 3.27, globulin 3.19; albumin 2.45, globulin 2.29. These limited observations indicate that the serum proteins are below normal in renal amyloidosis and not necessarily dependent upon edema. The heavy loss of protein in the urine would lead us to expect a depletion of the serum proteins.

Edema: Edema is a variable feature in amyloid renal disease. It was present at some time during the course of the disease in 32 of our 65 cases. It is apparently not causally related to the amyloid deposit since it is as frequent with minimum as with maximum amyloid accumulations (see Tables II–V).

Fahr [11] has also noted that there is no connection between the severity of the glomerular lesions and the edema. In a group with relatively slight renal changes edema was present in 4 and absent in 5; in those with severe changes it was present in 12 and absent in 4; in those with amyloid contracted kidneys it was present in 3 and absent in 2.

In 30 of our cases of chronic suppurative diseases, the majority of which had amyloid in the liver and spleen but none of which had amyloid in the kidneys, edema was absent in 12, slight in 12, moderate in 3, and severe in 3. Edema is obviously not due to the amyloid deposit, although it may be related to the glomerular injury which accompanies amyloidosis. As in other forms of renal disease edema varies in intensity from time to time. Cardiac decompensation was a factor in causing edema in a few of our cases.

Hematuria: A mild hematuria is occasionally found in amyloid disease (Bannick and Barker) but apparently it is seldom as pronounced as in acute glomerulonephritis. The erythrocytes escape chiefly from injured capillaries which do not contain amyloid.

Hypertension: The great majority of investigators find a low blood pressure in amyloid disease of the kidneys. Fahr [5] reported 6 cases with little or no nitrogen retention in which the blood pressure was low. McElroy [12] found the blood pressure 105/70 and the urea nitrogen 9.5 mg. in his patient, a woman 33 years of age. Lemierre and his coworkers [9] reported a constantly low blood pressure in a woman 32 years of age who died of uremia. Bannick and Barker,[2] in a case of Hodgkin's disease with amyloidosis in a male 38 years of age, found the blood pressure 100/70 to 115/75. The blood urea was 58 mg. and the phenolsulphonephthalein output 15 per cent in one hour. The patient of Linder, Maxwell and Green,[10] a boy 13 years of age, had a blood pressure of 112/72. The blood urea reached 199 mg. Zadek [13] reported 3 cases with uremia: a woman aged 52 years, blood pressure 130/78, blood urea 148 mg.; a male aged 63 years, blood pressure 120/60, blood urea 138 mg.; and a male aged 58 years, blood pressure 100/60, blood urea 159 mg. Rosenberg [14] states that the amyloid kidney is characterized by the absence of hypertension and cardiac hypertrophy.

However, there are a few reports of hypertension in amyloid renal disease. Fahr [15] mentioned 4 cases with slight hypertension. Danisch [16] reported a blood pressure of 173/101 in a male 65 years of age. The non-protein nitrogen was 141.3 mg. Noble and Major,[17] in a report of 3 cases with uremia, found 1 with a definite elevation of blood pressure. These 3 cases are included in Table V.

Referring to Table II it is seen that there are 2 instances of moderate hypertension in Group A. One of these shows a definite arteriolosclerosis and is therefore presumably a case of primary hyperten-

TABLE II

Group A. Cases in Which Only a Few Glomerular Capillaries are Filled with Amyloid

Autopsy No.	Sex	Age	Disease	Albuminuria	Edema	Blood pressure	Heart	Kidneys	Amyloid in glomeruli	Amyloid in arterioles	Amyloid in medulla	Casts	Urea nitrogen, mg. per 100 cc.	Phenolsulphonephthalein, % 2 hrs.	Comments
		yrs.					*gm.*	*gm.*							
12–15	M	49	Tuberculosis of lungs	1	–	0	200	240	1	1	–	1			
15–261	F	30	Tuberculosis of spine	–	–	0	151	220	1	1	–	–			
15–282	M	24	Tuberculosis of lungs	1	1	0	142	353	1	–	–	1			
15–283	M	36	Tuberculosis of lungs	1	–	0	235	normal	1	–	2	1			
21–494	M	13	Tuberculosis of spine.....	2	–	0	small	small	1	–	–	–			
22–74	F	58	Ulcerative enteritis	2	–	0	275	200	1	1	1	–			
23–29	M	36	Chronic abscess	0	–	0	240	370	1	1	1	–			
23–255	F	40	Tertiary syphilis ..... (hypertension)	3	3	152/94	270	590	1	1	0	2		25	arteriolosclerosis
25–508	M	36	Chronic osteomyelitis	3	–	0	300	625	1	–	–	–			
26–420	M	75	Tuberculosis of lungs	1	–	0	340	505	1	–	–	–			
26–652	F	28	Tuberculosis of lungs	1	2	110/64	200	325	1	1	1	–	23·3		
27–610	F	32	Tuberculosis of spine	4	1	80/50	140	300	1	1	2	–			
28–717	M	15	Transverse myelitis ..... (secondary infection)	3	–	0	normal	abscesses	1	–	–	–			
29–1106	F	41	Tuberculosis of lungs	2	1	118/78	160	215	1	1	–	–			
29–1642	M	43	Tuberculosis of lungs	4	–	82/40	220	360	1	–	–	–			
30–65	M	16	Tuberculosis of spine.....	1	1	0	150	280	1	1	–	–			
31–405	M	40	Perinephritic abscess.....	2	2	145/90	500	255	1	1	2	1	70		only one kidney
31–820	M	43	Tuberculosis of lungs.....	0	1	128/60	342	407	–	–	1	–			
31–1559	M	39	Tuberculosis of lungs.....	–	1	120/80	215	350	1	1	1	1			
32–426	M	33	Perinephritic abscess	2	3	126/72	115	250	1	–	–	1	10		

The numerals 1, 2, 3 and 4 under the several headings in this and the following tables indicate roughly the degree of the process. 0 indicates that no observation was made. – indicates absence of the condition indicated.

0 indicates that no observation was made. – indicates absence of the condition indicated.

TABLE III

Group B. Cases with Moderate Amyloid Deposits without Complete Obstruction of Glomeruli and Showing No Tubular Atrophy

Autopsy No.	Sex	Age	Disease	Albuminuria	Edema	Blood pressure	Heart	Kidneys	Amyloid in glomeruli	Amyloid in arterioles	Amyloid in medulla	Casts	Urea nitrogen, mg. per 100 cc.	Phenolsulphonephthalein, % 2 hrs.	Comments
		yrs.					*gm.*	*gm.*							
20–367	M	25?	Empyema	3	–	0	170	355	2	1	2	1			
21–52	F	26	Chronic osteomyelitis	3	3	120/82	340	680	2	2	–	2	norm.	50	
21–523	F	16	Tuberculosis of spine	2	–	0	very small	large	2	2	–	1			
24–512	M	28	Tuberculosis of lungs	3	–	0	200	560	2	2	1	1			
25–1045	M	23	Tuberculosis of spine	1	–	0	normal	normal	2	2	3	2			
26–134	F	15	Tuberculosis of hip	2	–	0	small	normal	2	1	1	–			
29–652	M	78	None	1	–	150/94	350	170	' 2	' 2	' 1	' 1			arteriolosclerosis
30–214	F	22	Chronic abscess	3	3	0	190	300	2	–	–	1			
30–1203	M	66	Tertiary syphilis	2	–	140/90	400	215	2	2	–	1			
30–1295	F	42	Chronic abscess	2	–	100/96	300	465	2	1	0	–			
30–1305	F	29	Empyema	0	3	120/88	not examined	294	2	1	1	1	norm.	norm.	
30–1752	M	48	Tuberculosis of lungs	3	–	86/60	218	260	2	2	3	3	29.6		
31–291	M	47	Chronic osteomyelitis	3	1	85/60	310	370	2	4	0	4	118		
31–1208	M	21	Unresolved pneumonia	–	–	116/72	300	360	2	–	0	–	17.7		
31–1356	F	35	Tuberculosis of lungs	4	–	95/68	340	370	2	2	1	1			

TABLE IV

Group C. Cases Showing Massive Deposits of Amyloid in Glomeruli with Obstruction of Capillaries and Showing Slight Tubular Atrophy

Autopsy No.	Sex	Age	Disease	Albuminuria	Edema	Blood pressure	Heart	Kidneys	Amyloid in glomeruli	Amyloid in arterioles	Amyloid in medulla	Casts	Urea nitrogen, mg. per 100 cc.	Phenolsulphonephthalein, % 2 hrs.	Comments
		yrs.					*gm.*	*gm.*							
13–16	F	31	Tuberculosis of lungs	0	–	0	not examined	small	3	3	3	–			
15–41	M	34	Tuberculosis of lungs	0	–	0	293	437	3	3	–	3			
20–451	M	12	Tuberculosis of lungs	3	–	0	145	325	3	1	1	1			
24–572	F	14	Chronic abscess	3	–	0	normal	large	3	1	2	3			
27–806	F	25	Empyema	3	2	0	223	445	3	1	0	2		15% (1hr.)	
28–145	M	48	Tuberculosis of lungs	0	–	0	233	505	3	1	3	1			
28–322	M	51	Pyonephrosis	3	1	110/60	350	700	3	2	–	1			
28–545	M	18	Transverse myelitis (secondary infection)	4	1	0	normal	very large	3	2	3	2			
29–1804	F	50	None ?	1	2	170/120	435	215	3	3	–	–	52.5		
30–533	M	23	Tuberculosis of spine	2	1	110/70	300	800	3	1	1	4			
30–982	M	46	Pyonephrosis	4	1	98/48	not examined	500	3	1	0	3	121	0	
30–1389	M	33	Tuberculosis of lungs	4	1	105/72	315	435	3	2	0	1	178		
30–1572	M	49	Tuberculosis of lungs	3	–	136/104	505	652	3	–	0	1	65		
31–1754	M	46	Empyema	3	1	238/142	375	325	3	3	1	3			
31–1877	M	43	Tuberculosis of lungs	2	–	90/58	300	380	3	3	–	4			
31–1934	F	47	Chronic arthritis	3	4	120/70	200	220	3	2	1	2	9.4	22	congo red, 56 % retention
31–2015	M	6	Tuberculosis of spine	2	4	0	72	185	3	–	2	–			endocarditis
32–362	M	43	Tuberculosis of lungs	4	3	110/90	265	400	3	1	1	–			

TABLE V

Group D. Cases Showing Extreme Glomerular Obstruction with Amyloid and Marked Tubular Atrophy

Autopsy No.	Sex	Age	Disease	Albuminuria	Edema	Blood pressure	Heart	Kidneys	Amyloid in glomeruli	Amyloid in arterioles	Amyloid in medulla	Casts	Urea nitrogen, mg. per 100 cc.	Phenolsulphonephthalein, % 2 hrs.	Comments
		yrs.					*gm.*	*gm.*							
16–45	M	61	Tuberculosis of lungs	2	2	0	550	very small	4	4	2	2			mitral defect
20–84	F	59	Tertiary syphilis	0	1	0	300	520	4	1	2	4			
21–457	M	59	Tuberculosis of lungs	3	–	0	275	370	4	4	0	1			
25–204	M	19	Tuberculosis of hip	1	3	0	normal	large	4	2	1	1			
28–64	F	35	None	3	–	190/110	355	260	4	3	0	1	147		
28–217	M	58	Bronchiectasis	2	–	0	435	240	4	1	2	4			
28–1214	M	15	Chronic osteomyelitis	3	–	0	normal	large	4	2	2	2			
28–1616	M	55	None	4	1	104/50	480	280	4	4	4	–	134		
28–1629	M	27	Chronic osteomyelitis	4	1	110/?	220	575	4	3	1	4	68	0	
29–1425	M	54	Ulceration of legs	4	–	120/60	580	440	4	4	2	4	134.6	0	
32–52	M	43	Tuberculosis of lungs	–	–	0	375	370	4	2	3	1			
32–175	M	75	Tertiary syphilis	4	4	110/64	not examined	240	4	2	–	1	N.P.N. 58.8		

sion. In the other case (31–405) no cause for the cardiac hypertrophy was found. It cannot be attributed to the amyloid deposit since this was minimal in amount. In Group B 1 instance of moderate hypertension was found which was presumably due to arteriolosclerosis and not to amyloid. In Groups C and D, that is, those with severe changes in the glomeruli, there are 3 cases of hypertension among 15 in which the blood pressure was recorded. In all 3 of these there are massive deposits of amyloid in the arterioles which make it impossible to decide whether there are any hyaline changes characteristic of primary hypertension. A summary of the clinical histories of these 3 cases is as follows:

29–1804 (Table IV, Group C): A woman 50 years of age was admitted to the hospital Nov. 19, 1929, complaining of dyspnea and moderate generalized edema. Dyspnea with a constant non-productive cough had been present for about one year, and edema about two months. There was slight cyanosis of the face, a slight bilateral exophthalmos, and a bilateral lid lag. The thyroid was not palpable. A few moist râles were heard in both lung bases. The heart was enlarged to the left, and a systolic murmur was heard at the apex. The abdomen was distended and a fluid wave was elicited. The blood pressure was 170/120. Urinalysis on many different examinations showed a trace of albumin, hyaline casts, and sometimes erythrocytes. Hemoglobin was 77 per cent; erythrocytes 4,350,000, leukocytes 6100, blood sugar 0.07 per cent; creatinin 1.8 mg.; urea nitrogen 52.5 mg.; blood Wassermann ++++. Death occurred Dec. 11, 1929. No anatomical evidence of syphilis was found at postmortem, but the positive Wassermann reaction suggests syphilis as a possible factor in causing the amyloid disease. There was no other assignable cause for the amyloidosis. There was no amyloid in the liver or spleen.

31–1754 (Table IV, Group C): A male 46 years of age developed empyema following pneumonia in 1919. Right thoracoplasty was performed. Four years later a sinus developed which has persisted, having been treated by irrigations. His last illness began Oct. 11, 1931, with deep-seated pain in the right chest. On October 21 he became semicomatose. On October 22 he was admitted to the hospital in coma. He had one convulsion. His muscles were spastic and the reflexes were hyperactive. The temperature was 105° F; leukocyte count 26,000; albuminuria +++; blood pressure 238/142. Death occurred Oct. 24, 1931. No determination of blood urea was made. The changes found in the kidneys support the clinical diagnosis of uremia. There was a residual empyema in the right chest.

28–64 (Table V, Group D): The patient, a woman 35 years of age, was admitted to the hospital Dec. 13, 1927, complaining of impairment of vision, pain in the chest, dyspnea and frontal headache. These symptoms had begun about two weeks previously. The heart was enlarged to the left and there was a blowing systolic murmur over the apex transmitted to the axilla. The blood pressure was 190/110. A few coarse râles were heard posteriorly over the bases of the lungs. The liver was definitely palpable below the right costal margin. There was slight edema of the legs. The blood Wassermann reaction was negative. The urine showed albumin from + to ++++. December 19, creatinin 3 mg.,

urea nitrogen 116 mg. December 31, creatinin 3 mg., urea nitrogen 147 mg. She had a slight fever during the last three days of life. Ophthalmoscopic examination revealed an albuminuric retinitis. Death occurred Jan. 10, 1928. The clinical diagnosis was chronic glomerulonephritis, but the postmortem revealed advanced amyloid disease of the kidneys. No cause of amyloidosis was found. The clinical picture seems indistinguishable from chronic glomerulonephritis.

It may be concluded that there are occasional instances of renal amyloidosis in which a definite hypertension develops that is presumably due to amyloid which causes narrowing of the glomerular capillaries and the arterioles of the kidneys. It is known that increased resistance in the renal circulation may cause hypertension.

Renal Insufficiency in Amyloid Disease: It is well known that amyloid disease may terminate in uremia. Cases of this type have been reported by Danisch (2 cases), Linder, Maxwell and Green (1), Noble and Major (3), Lemierre and associates (1), and Zadek (3).

In our series functional tests were made on only 20 of the 65 patients. The patient in Group A (Table II, 31–405) with a urea nitrogen of 70 mg. had only one kidney and it was surrounded by an abscess. The case in Group B (Table III, 31–291) with a urea nitrogen of 118 mg. showed only a moderate amyloid deposit, but the tubules were nearly all blocked by casts. All the cases in Groups C and D that were studied show more or less evidence of impaired kidney function. A histological study of the kidneys of these two groups reveals extensive obliteration of glomerular capillaries, which is convincing evidence of a marked impairment of kidney function. All except 3 of these patients were suffering from a well defined chronic infectious process, yet renal insufficiency must have played an important rôle in causing death.

Size of the Kidneys: In amyloid disease the kidneys are usually larger than normal, but they may be of normal size or contracted. In 41 of 72 cases Raubitschek found "large white kidneys." The average weight of both kidneys in our series was as follows: Group A, 349 gm.; Group B, 366 gm.; Group C, 452 gm.; and Group D, 366 gm. The enlargement is due in part to cloudy swelling resulting from the infection, since about half the kidneys in Groups A and B, with a relatively small amount of amyloid, are above normal size. The largest kidneys are found in Group C, in which there is the greatest amount of amyloid, but even in these swelling of the epithelial cells and dilatation of the tubules is chiefly responsible for the

increased size. In Group D with uremia there are some small kidneys, but some are large and we do not find the extremely contracted kidneys that are often seen in long-standing glomerulonephritis. It is clear that uremia may develop before the kidneys become contracted.

Alterations in the Glomeruli: The glomerulus is nearly always involved, even in the earliest stages of renal amyloidosis. In our 65 cases there was only one instance in which the glomeruli were unaffected and in this there was only a minimal deposit in the medulla. The disturbances of renal function are caused chiefly by obstruction of the glomerular capillaries.

In the older literature the opinion was frequently expressed that amyloid is deposited in the capillary wall. Hueter,[18] in 1908, one of the first investigators to demonstrate the capillary basement membrane, observed that the first deposits of amyloid are on the inner surface of this structure. Ohmori,[19] in 1921, came to the same conclusion. My observations are in full accord with those of Hueter and Ohmori. In sections stained with azocarmine the first accumulations of amyloid are readily seen on the inner surface of the basement membrane, *i.e.*, in the lumen of the capillary (Figs. 1 and 2). At no time is amyloid found external to a demonstrable basement membrane. In the early stages it is usually possible to demonstrate the membrane distinctly from the amyloid, but frequently the amyloid blends with the membrane from the first (Fig. 3). When the capillary is well filled the distinction between membrane and amyloid is usually lost (Fig. 4), but sometimes the membrane is demonstrable in capillaries distended with amyloid (Fig. 5).

An increase of endothelial nuclei in amyloid disease was noted by Hueter, and Fahr also observed this change in some instances. In our material there is a convincing increase of capillary endothelial cells in over 50 per cent of the kidneys. This occurs in capillaries without amyloid, as well as those that contain amyloid. It is also found frequently in kidneys of persons dead of chronic infectious diseases in which there was no renal amyloidosis. Severe infections of various kinds, notably subacute bacterial endocarditis, usually cause an increase of glomerular endothelium (Bell[20]). This endothelial proliferation is interpreted as acute glomerulitis. It is often quite prominent in amyloid disease (Fig. 1), but in no instance did it reach the degree characteristic of clinical acute glomerulonephritis.

It is obvious that endothelial proliferation is caused by the associated infection and not by the amyloid deposit.

The first accumulation of amyloid is on the inner surface of the basement membrane. If any endothelial nuclei are present at the site of the amyloid deposit they are usually, but not always, displaced toward the lumen of the capillary (Fig. 2). When the capillary is filled by an accumulation of amyloid on one side, the nuclei of the opposite side are left in contact with the basement membrane. In capillaries greatly distended with amyloid the nuclei are frequently scattered throughout the amyloid deposit (Fig. 5).

Raubitschek [1] noted that amyloid may accumulate without narrowing the lumen of the capillary, and Hueter observed that this was due to a simultaneous enlargement of the capillary which may attain two or three times its normal diameter. Fahr [5] commented on the remarkable permeability of the glomerulus that may be found in advanced amyloidosis. The enlargement of the individual capillaries explains the markedly enlarged glomeruli that are frequently seen. In such large glomeruli there may be a good capillary circulation, the blood being in direct contact with the amyloid deposit. Frequently glomeruli of normal size are seen in which all the capillaries are filled with amyloid — in these no stage of enlargement has occurred. Rarely all the glomeruli are of this type. Ultimately the glomerular circulation is completely blocked by the amyloid deposit, and atrophy of the associated tubule begins. The completely obstructed glomerulus gradually shrinks in size and loses the capacity to react to the specific amyloid stain. Finally it has a homogeneous structure and gives no amyloid reaction (Fahr). Our observations agree with those of Hueter that the glomerular epithelium is largely desquamated in advanced amyloid disease of the glomerulus.

The hyaline glomeruli of the amyloid kidney differ fundamentally from those of chronic glomerulonephritis in that the hyaline is derived from amyloid and not from intracapillary fibers. They also differ sharply from the hyaline glomeruli of primary hypertension which form by fusion of thickened capillary basement membranes.

Casts: Casts play an important rôle in the amyloid kidney. Their prominence usually corresponds to the degree of renal amyloidosis (see Tables II–V). They do not give the amyloid reaction, but apparently are of firmer structure than in other diseases, since they have a much greater tendency to lodge in the tubules. Obstruction

of the lumen causes dilatation and sometimes atrophy of the tubule. Fahr lays great stress on casts as a cause of destruction of the kidney and renal insufficiency. They are often a factor in renal insufficiency and in 1 instance (Table III, Group B, 31–291) they were more important than the glomerular lesion in causing uremia. However, they are seldom the cause of extensive tubular atrophy.

The Arterioles: Hueter thought that the arterioles were frequently involved before the glomeruli, but in our material the deposit seems to occur at about the same time in arterioles and glomeruli (Tables II–V). The amyloid accumulates in the media of the arterioles between the individual muscle cells (Raubitschek). In this respect it differs clearly from the subintimal hyaline deposit found in hypertension. An early amyloid involvement of an arteriole is readily distinguished from hypertension, but a massive amyloid deposit may obscure the evidences of hypertension. The involvement of the arterioles is often a very prominent feature in advanced amyloid disease (Noble and Major [17]). There is some narrowing of the lumens of the diseased arterioles, and there is presumably a loss of vasomotor responses. No doubt arteriolar disease is a factor in the destruction of the kidney.

The Medulla: One of the most frequent sites of the amyloid deposit is the medulla (Tables II–V). This seems to take place at about the same time as the deposit in the glomeruli and arterioles. The amyloid accumulates chiefly in the walls of the small vasa recta, displacing the muscular layer. Occasionally there is an accumulation under the basement membrane of the straight tubules or in the interstitial connective tissue. Rarely there is sufficient amyloid in areas of the medulla to compress the tubules and contribute to tubular atrophy.

The Tubules: In two instances in this series the cortical tubules were extensively destroyed by deposits of amyloid under the basement membrane, but this feature was unimportant in the rest. Fahr attaches great importance to tubular degeneration in the destruction of the amyloid kidney, although he concedes that the glomeruli are the chief cause of tubular atrophy. He believes that the tubules may be destroyed by distention from casts and by hyaline granular degeneration. He describes necrotic tubules. In well fixed material we have never observed necrotic tubular epithelium. Hyaline granular degeneration was seen in about half the kidneys of Groups A, B and

C, and in about one-third of those of Group D. This type of degeneration does not seem to progress as far as necrosis of the cell. Fahr also observed that hyaline granular degeneration was less frequent in uremic amyloid kidneys.

Tubular atrophy is usually conspicuous in advanced amyloid disease. The atrophic tubules are nearly always associated with glomeruli obstructed by amyloid. Rarely the atrophy is to be attributed to obstruction by casts or by amyloid in the medulla, and rarely to direct compression of the tubule by amyloid.

The Relation of Amyloid Renal Disease to Glomerulonephritis: In the older literature, before the different types of nephritis were sharply defined, the prevailing opinion was that amyloid disease is not a special type of nephritis but a complication of some form of preexisting renal disease. Raubitschek [1] stated that in a group of 72 cases of renal amyloidosis there were only 13 examples of pure amyloid disease without inflammatory changes, and that in 1 instance an acute nephritis was present. Hueter [18] stated that a nephritis usually precedes amyloidosis of the kidneys. MacCallum [21] seems still to hold this opinion, since he states that amyloid is only an incidental deposit which may modify the course of a nephritis but does not cause it.

In the more recent literature the amyloid kidney is usually interpreted as a primary renal disease, but a few authors interpret it as a complication of a preëxistent lipoid nephrosis or glomerulonephritis. Fahr [22] classifies amyloid disease as a special form of nephrosis. He recognized a definite increase of endothelial nuclei in 10 of 40 cases, a pronounced proliferation in 2 of these; but he considers the endothelial proliferation a secondary phenomenon and not evidence of a true glomerulonephritis. The disease is classified with the nephroses because the lesions are all interpreted as degenerative and not inflammatory in nature. Fahr recognizes the close clinical resemblance of some cases of amyloid disease to lipoid nephrosis, but he apparently is not convinced that amyloid is superimposed on lipoid nephrosis.

It is frequently difficult to distinguish amyloid renal disease clinically from glomerulonephritis. Some cases resemble the azotemic type, others the hydropic form of glomerulonephritis (lipoid nephrosis). This similarity is evident in a number of recent reports.

Bannick and Barker studied a case of Hodgkin's disease in a male

38 years of age. There was a marked albuminuria and at one time a mild hematuria. The blood pressure was 100/70 to 115/75. There was no edema. There was some impairment of renal function: phenolsulphonephthalein 15 per cent in one hour, blood urea 58 mg. The total serum protein was 3.8 gm., of which 27 per cent was albumin. Cholesterol was 235 mg. The diagnosis of amyloid disease was established by the Congo red test.

Lemierre and colleagues described a case of renal amyloidosis resulting from pulmonary tuberculosis. Albuminuria was present during a period of two years, but there was never any edema. The last determination of serum proteins was globulin 3.19 mg., albumin 2.45 mg. Blood urea was 30 to 38 mg. until toward the end of life when it rose to 124, 237 and 369 mg. The blood pressure was always low.

Linder, Maxwell and Green reported an example of amyloid renal disease in a boy 13 years of age. A mastoid operation at the age of 4 years was followed by the formation of a sinus which discharged from time to time. In 1925 edema and proteinuria were noted. Oct. 29, 1925, he had albuminuria, general anasarca, and blood pressure 112/72. The edema disappeared in December, 1925 and did not recur. The blood urea rose to 199 mg. Blood pressure never increased. The serum proteins varied from 3.8 to 4.7 gm. The cholesterol was 920 mg. in May 1926, but it decreased during the uremia. Death occurred in August, 1926.

Danisch [16] reported an example of amyloid disease in a man 65 years of age. Swelling of the feet was first noticed in 1917. In the fall of 1921 the swelling increased. He was confined to bed from January to March, 1922. In August, 1923, he had heavy albuminuria, very marked edema, and a blood pressure of 173/101. The blood pressure remained high. In December, 1924, the non-protein nitrogen was 141.3 mg. Death occurred in December, 1924. Contracted amyloid kidneys were found postmortem.

In several of our cases the resemblance to chronic glomerulonephritis is obvious. No. 31–1934, Group C, was regarded clinically as the hydropic type of glomerulonephritis until the Congo red test was made. The associated chronic arthritis, of course, suggested the possibility of amyloid disease. The same clinical diagnosis was made in No. 32–175, Group D. No. 28–64, Group D, was diagnosed clinically as the azotemic form of chronic glomerulonephritis. Amyloid disease was not suspected since there was no associated infection.

When an infectious process of long duration is present, one should think first of amyloid disease as an explanation of renal symptoms. When there is no known cause for amyloidosis the true nature of the renal lesion may not be suspected. An enlarged firm liver suggests amyloid. The Congo red test is of great value in many instances.

Amyloidosis is a special type of renal disease. In some instances it exhibits the clinical features considered characteristic of nephrosis and in others it presents the phenomena regarded as typical of nephritis. Occasionally the disease progresses from a picture of nephrosis to one of nephritis. It does not clarify our conceptions to force it into the group of nephroses, especially since the distinction between nephrosis and nephritis is becoming less sharp as our knowledge of renal disease increases. It is true that the renal lesions are chiefly degenerative in character, but there is commonly a definite endothelial proliferation at the onset of the disease.

Amyloidosis is a primary disease of the kidney in the sense that it is rarely superimposed on any clinical form of renal disease. At the onset there is commonly a mild acute diffuse proliferative glomerulitis caused by the infection responsible for the amyloidosis, but this inflammatory reaction does not attain the intensity characteristic of clinical acute glomerulonephritis, and it is frequently absent entirely. We have one example of amyloidosis superimposed on a hypertensive kidney, but the renal disturbance in this instance was largely caused by the amyloid deposit. A combination of amyloid with chronic glomerulonephritis seems possible, but we have not seen such a case.

Summary

A study of 65 cases of amyloid disease of the kidneys is reported. These are arranged in four groups corresponding roughly with the degree of glomerular involvement.

In Groups A and B the symptoms are essentially those of the underlying infection, and with few exceptions albuminuria or edema is the only symptom referable to the kidneys.

In Group C there is some impairment of renal function, and in Group D there is evidence of advanced renal insufficiency.

Albuminuria is rarely absent, but the amount of albumin does not indicate accurately the extent of the amyloid deposit.

Edema is a variable feature with no evident relation to the degree of renal damage.

Hypertension is occasionally found in amyloid disease with renal insufficiency. It is probably due to obstruction in the arteriolar and glomerular circulation.

Renal insufficiency is a frequent cause of death. It is caused chiefly by amyloid deposits in the glomerular capillaries, but obstruction of the tubules by casts and amyloid deposits in the medulla, around the tubules and in the arterioles, are often important factors in the production of uremia.

In the glomerulus amyloid is deposited on the inner surface of the capillary basement membrane. Endothelial nuclei are frequently displaced inwardly and become scattered through the amyloid. The capillaries usually become greatly distended with amyloid and they may remain permeable in the presence of massive deposits. The glomerular epithelium degenerates and is desquamated.

There is commonly a definite increase of endothelial nuclei in the glomerular capillaries preceding the deposit of amyloid. This is attributed to the underlying infection. It is not sufficiently prominent to be identified with clinical acute glomerulonephritis.

Amyloidosis is a special form of renal disease. There is no advantage in classifying it as a nephrosis. A sharp distinction between nephrosis and nephritis has not been established.

Amyloidosis is a primary renal disease. It is rarely a complication of a preëxistent clinical renal lesion.

REFERENCES

1. Raubitschek, H. Über Nierenamyloidose. *Virchows Arch. f path. Anat.*, 1905, **182**, 297–313.
2. Bannick, E. G., and Barker, N. W. Diffuse amyloidosis of unknown etiology. *M. Clin. N. Amer.*, 1930, **14**, 773–781.
3. Magnus-Levy, A. Bence-Jones-Eiweiss und Amyloid. *Ztschr. f. klin. Med.*, 1931, **116**, 510–531.
4. Weber, H. Amyloidablagerung in einem multiplen plasmacellulären Myelom. *Beitr. z. path. Anat. u. z. allg. Pathol.*, 1931, **86**, 1–14.
5. Fahr, Th. Über Nephrose. *Deutsches Arch. f. klin. Med.*, 1918, **125**, 66–106.
6. Ekehorn, G. Principles of renal function. *Acta med. Scandinav.*, 1931, *Suppl.* **36**, 1–717.
7. Geill, T. Klinische Untersuchungen über Albumin und Globulin im Blut und im Harn bei Nierenkranken. *Ztschr. f. klin. Med.*, 1929, **110**, 334–381.

8. Hiller, A., McIntosh, J. F., and Van Slyke, D. D. Excretion of albumin and globulin in nephritis. *J. Clin. Investigation*, 1927, **4**, 235–251.

9. Lemierre, A., Léon-Kindberg, Cottet and Mahondeau-Campayer, D. Sclérose amyloïde du rein à forme albuminurique simple. Azotémie aiguë terminale. *Bull. et mém. Soc. méd. d. hôp. de Paris*, 1931, **47**, 1769–1779.

10. Linder, G. C., Maxwell, J., and Green, F. H. K. Clinical, pathological, and biochemical study of amyloid nephrosis. *Arch. Dis. Childhood*, 1927, **2**, 220–230.

11. Fahr, Th. Beiträge zur Frage der Nephrose. *Virchows Arch. f. path. Anat.*, 1922, **239**, 32–40.

12. McElroy, J. B. Nephroses. *J. A. M. A.*, 1927, **89**, 940–945.

13. Zadek, E. Uber Urämie bei Amyloidniere. *Klin. Wchnschr.*, 1931, **10**, 827–828.

14. Rosenberg, M. Zur Klinik der Amyloidniere. *Deutsche med. Wchnschr.*, 1931, **57**, 99–101.

15. Fahr, Th. Zur Frage der Amyloidnephrose und Amyloidschrumpfniere. *Klin. Wchnschr.*, 1931, **10**, 1205–1208.

16. Danisch. Über Amyloidschrumpfnieren. *Verhandl. d. deutsch. path. Gesellsch.*, 1925, **20**, 307–318.

17. Noble, J. F., and Major, S. G. Renal insufficiency in amyloid disease. *Arch. Path.*, 1929, **8**, 762–774.

18. Hueter, C. Ueber Amyloid der Glomeruli. *Centralbl. f. allg. Pathol. u. path. Anat.*, 1908, **19**, 961–965.

19. Ohmori, D. Bedeutung der Membrana propria der Glomerulusschlingen in der Nierenpathologie. *Virchows Arch. f. path. Anat.*, 1921, **234**, 53–72.

20. Bell, E. T. Glomerular lesions associated with endocarditis. *Am. J. Path.*, 1932, **8**, 639.

21. MacCallum, W. G. A Text-book of Pathology. W. B. Saunders Co., Philadelphia, 1924, Ed. 3, 297.

22. Fahr, Th. Pathologische Anatomie des Morbus Brightii. Handbuch der speziellen pathologischen Anatomie und Histologie. J. Springer, Berlin, 1925, **6**, 156.

DESCRIPTION OF PLATE

PLATE 30

The drawings were all made from preparations stained with azocarmine (Mallory-Heidenhain stain). Each represents a small portion of a glomerulus: am. = amyloid deposit, b.m. = basement membrane, end. = endothelial nucleus, ep. = glomerular epithelial cell, er. = erythrocyte. × 1000.

FIG. 1. Capillaries showing the initial stage of amyloid disease. The amyloid is deposited on the inner surface of the basement membrane. The membrane is indistinct under one of the amyloid masses. There is a marked increase of endothelial cells.

FIG. 2. Capillaries showing a moderate amount of amyloid. The basement membrane is everywhere distinct. There is an increased number of endothelial nuclei, and some of them are displaced centrally. The epithelial cells are largely desquamated.

FIG. 3. Capillary illustrating a type in which the initial deposit of amyloid blends with the basement membrane.

FIG. 4. Capillary containing a large amyloid deposit. Only a small lumen persists. The basement membrane is no longer demonstrable under the larger amyloid masses. There is a definite increase of endothelial nuclei, and some of them are still in contact with the basement membrane.

FIG. 5. Capillary greatly distended with amyloid. The basement membrane is distinct. Small spaces persist through which the blood still circulates. There is an increased number of endothelial nuclei. This type of capillary is found in the very large glomeruli.

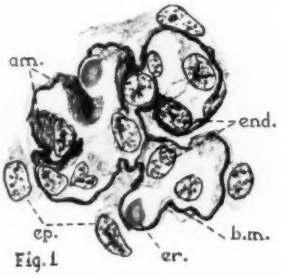


Fig. 1

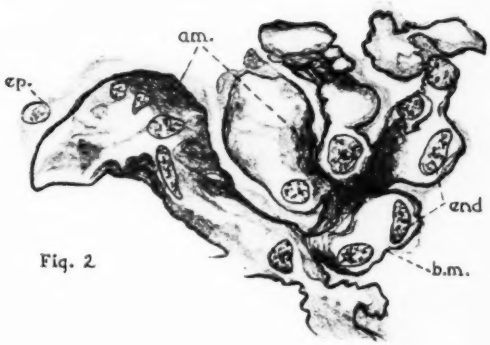


Fig. 2

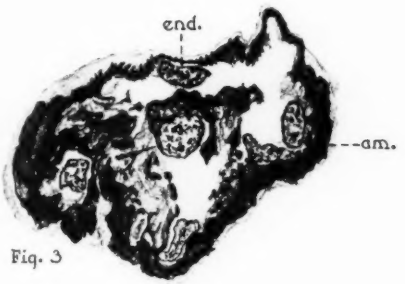


Fig. 3

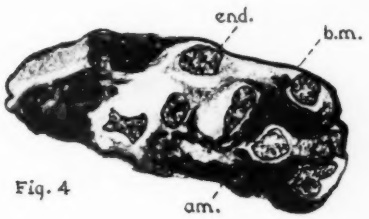


Fig. 4

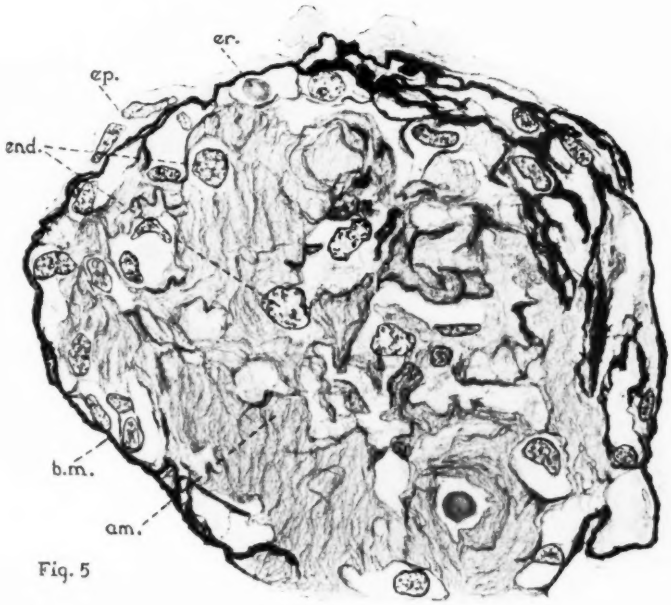


Fig. 5

THE RELATION BETWEEN THE MITOCHONDRIA AND GLUCOSE-GLYCOGEN EQUILIBRIUM IN THE LIVER *

ERNEST M. HALL, M.D., AND EATON M. MACKAY, M.D.

(From the Department of Pathology of the University of Southern California School of Medicine, Los Angeles, and the Scripps Metabolic Clinic, La Jolla, California)

In recent years considerable work has been undertaken in an effort to discover the rôle of the mitochondria (chondriosomes) of the hepatic cell, especially in relation to the glycogen cycle. This relationship has been variously interpreted by different investigators. The reason for the wide variation in interpretation is, no doubt, largely due to the fact that the function of these very minute bodies must of necessity be interpreted in terms of changes in morphology and distribution within the cell. Obviously such interpretations are little more than conjectures unless they are supported at every stage by experimental data.

The literature on the subject of mitochondria is so vast that we shall refer only to papers that deal directly with the relation of mitochondria to the glucose-glycogen cycle. Arnold [1] in 1908 found hypertrophy of mitochondria under certain conditions. He concluded that this increase in size is directly proportional to the amount of glycogen in the liver and assumed that the swelling of the chondriosomes is due to glycogen accumulating in them in the form of drops (Tropfen). Bang and Sjövall,[2] and Noël [3] state that the distribution of glycogen within the cytoplasm of the cell does not conform to the distribution of the mitochondria. Noël, after careful and extensive study, concluded that the cells from the morphological standpoint appeared to be the seat of two separate or independent processes, "les processus mitochondriaux et le processus glycogenique." The location of the glycogen and the chondriosomes, glycogen in spaces limited by the protoplasmic trabeculations, and chondriosomes in the trabeculations, makes it difficult to understand that there is any connection between the two. Mann [4] in 1928 reviewed the subject of mitochondria in glycogen relationship and concluded that none exists.

* Received for publication September 15, 1932.

Kater [5] has recently pointed out that all observers heretofore had attempted to correlate mitochondrial morphology with static aspects of glycogen deposition and suggested the desirability of investigating the morphology of the mitochondria of the hepatic cell at a time when the glycogen-glucose equilibrium is known to be disturbed.

Kater's choice of a plan of experiment was rather unfortunate in so far as a test of the conditions he had in mind are concerned. No determinations were made, either chemical or histological, of the glycogen content of the livers, so that it was impossible to know in which direction or in what degree the glycogen synthesis-glycogen hydrolysis equilibrium was disturbed. Blood sugar determinations which were carried out are of little value in such a study because marked increases and decreases may occur without any change in the liver glycogen, or, as in the case of epinephrine, a high blood sugar may be accompanied by an increase in liver glycogen. Kater's animals were fasted for twenty-four hours, which would reduce the liver glycogen to a very low value and make variations in the equilibrium difficult to study. Some animals were kept under ether anesthesia for a long time, a condition under which the hepatic glycogen changes would at most be uncertain. He gave some animals insulin. Its action on glycogenolysis is very indefinite, although in time it definitely reduces liver glycogen. Epinephrine, which was also used to modify the equilibrium, has a variable action causing early increases and later decreases in glycogenolysis. In any case a knowledge of the time factor is necessary for the interpretation of the results.

Contrary to the results of most of the other investigators cited, Kater concluded that some relation exists between the mitochondria of the hepatic cell and the glucose-glycogen equilibrium. He found in cases of increased metabolic activity a marked tendency toward enspherulation and hypertrophy of mitochondria.

Since Kater's paper was published two of his pupils, Clark and Hair,[6] have reported their studies on the mitochondria of the hepatic cell of the frog in normal and hyperglycemic states. These authors believe that much of the variation of opinion on the subject at hand is due to failure of the various investigators to take cognizance of two phases of hepatic cell activity, namely, carbohydrate metabolism and bile secretion. They point out that bile secretion is closely related to the presence of food in the intestine and believe, therefore, that it is necessary to disturb the mechanism through

some other means than feeding in order to cause activity in only one phase.

These considerations led them to choose the frog in its dormant state as their experimental animal. Besides a control group of ten animals, three other groups were used as follows: one group was injected intramuscularly with adrenalin-hydrochloride solution, another group was given glucose, and a third group was etherized for two and one-half hours.

The authors found no disturbance of the morphology of the mitochondria obtained from the sugar injections. Adrenalin-hydrochloride injections and etherization caused increase in size and enspherulation of mitochondria, most marked about the central vein. They agree with Kater that there is some relation between the mitochondrial morphology of the hepatic cell and the glucose-glycogen equilibrium.

While the frog in its dormant state may present some advantages over other laboratory animals in a study of this character, it must be borne in mind that the mitochondria of the liver cell are extremely variable in morphology, as the authors themselves found in a study of their ten control animals. It should also be remembered that glycogen synthesis in the liver is far more active at night while no food is being ingested than it is during the daytime. It seems probable that bile secretion interferes but slightly with glycogen metabolism.

Material and Methods

Experiment 1. Carrot-Feeding Experiments: The remarkable deposition of glycogen in the liver, which we found to follow carrot feeding of rabbits,[7] first interested us in the problem being considered here. In view of Kater's work it seemed desirable to correlate this increase with the mitochondrial changes. Three groups, each composed of three young female rabbits, were placed upon diets of dried alfalfa, dried carrot and fresh carrot [7] respectively. Twelve days later and 8 hours after being fed, when they were full of freshly ingested food material (rabbits eat more or less steadily throughout this period), all of the rabbits were removed from their diets and one from each group killed immediately. Twelve hours later a second was sacrificed and after 24 hours the third of each group was killed. The chemical analyses are presented in Table I and depicted graphically in Text-Fig. 1.

The animals were killed by injecting 5–10 cc. of air into the marginal ear vein. Before either respiratory or cardiac movements had entirely ceased the liver was rapidly excised and samples simultaneously removed for chemical glycogen determinations and for fixation in the various solutions for mitochondria and glycogen stains. Thirty seconds sufficed for all of this. Great care was exercised in cutting and handling small bits of tissue for fixation because of the marked changes in the finer cell morphology associated with squeezing, drying, and so on. Samples for the glycogen determinations were weighed quickly to within 0.5 per cent and dropped into boiling 60 per cent KOH and the glycogen analyzed by a modification of Pflueger's method, determining the glycogen as glucose after acid hydrolysis in place of weighing the substance.

Histological Methods

In recent years a number of relatively simple methods for staining mitochondria have been worked out. We have used two of these methods with some success.

Small blocks of tissue taken with great care as described above were fixed in Regaud's formalin and bichromate mixture. Other blocks were fixed in Flemming's fluid in preparation for Benda's crystal violet alizarin method. Satisfactory results were not obtained by this method. Using the blocks fixed in formalin and potassium bichromate (Regaud) the iron hematoxylin method of Heidenhain gave a fairly good differentiation. Good results were also obtained by use of Cowdry's modification of Altmann's anilin fuchsin and methyl green method, employing the same fixation. This stain gave a slightly better differentiation than the iron hematoxylin method. By this method the mitochondria stain brilliant red while the nuclei are olive green.

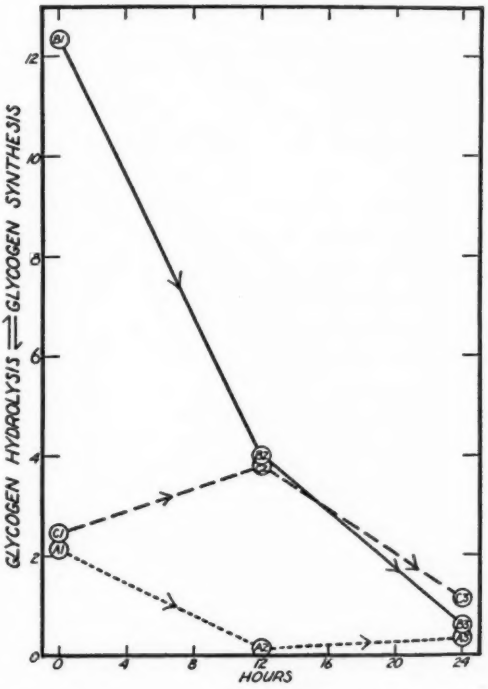
Small blocks, only 3–4 mm. square and about 2 mm. thick, were embedded in hard paraffin (56–58° C). Sections were cut 2 and 5 microns thick. The mitochondria were too dense for good differentiation in most of the 5 micron sections. Those cut at 2 microns were quite satisfactory and stained well.

Other small blocks of liver tissue were also taken for fixation in 95 per cent alcohol. These were embedded in celloidin and stained by Best's carmine method to show the relative amounts of glycogen present and its distribution within the liver lobules.

RESULTS

The results of Experiments 1, 2 and 3 are statistically and graphically shown in Tables I to III.

Experiment 1 (Table I): The effects of heavy carrot feeding on hepatic glycogen and mitochondria were studied in this experiment.



TEXT-FIG. 1. Graph (Experiment 1) showing changes in the percentage of hepatic glycogen plotted against time in hours under the various experimental conditions. Arrows directed away from the base line indicate synthesis of glycogen; those directed toward the base line indicate hydrolysis of glycogen.

Rabbit A1 fed on an ordinary diet of alfalfa served as a control. In the control the hepatic glycogen by chemical determination was slightly over 2 per cent. Sections stained by Best's carmine method show the cells of approximately the central two-thirds of the lobules filled with red granules. In Table I this amount is designated as

++. It will be seen, from a study of the various tables, that each plus sign, in a general way, corresponds to approximately 1 per cent glycogen by chemical determination. The histological examinations for glycogen were made independently of the figures obtained for the chemical determinations. With one or two exceptions the agreement between the two methods is very close. The diagrammatic drawing of the liver cell (A_1, Table I) shows that the mitochondria in the control liver are tiny bacilliform rods scattered indiscriminately throughout the cytoplasm (Fig. 1). Reference to Tables II and III shows the mitochondria of the other control animals very similar in morphology and distribution.

Rabbits A_2 and A_3, fasted for 12 and 24 hours respectively, showed marked reductions in the glycogen content of the livers by both chemical and histological methods. The mitochondria of the liver cells exhibit some tendency to become spherical or coccoid in form.

The changes produced in the liver cells of Rabbit B_1, fed on pulverized dried carrot, are the most outstanding of any in our series in regard to both glycogen content and transformation of mitochondria. The liver glycogen reached the astounding figure of 13.10 per cent, while every cell of the liver lobules is completely filled with coarse, red-stained granules of glycogen. In some of these cells the granules are so coarse that only 10–20 angular masses of glycogen can be counted in a single cell. The average is probably 30–35, while in the sections of the control liver (A_1) two to three times as many fine granules are present in each of the cells about the central veins. Corresponding with this great storage of glycogen profound changes are observed in the cells stained for mitochondria. The hepatic cells are markedly swollen and clear, except about the nuclei and cell peripheries where dark masses of mitochondria have accumulated (Fig. 2). In many cells the nucleus is almost obscured by the condensation of mitochondria upon it. Likewise scattered clumps of two or three up to a dozen or more mitochondria are piled up on the limiting membrane of the cell. The masses of mitochondria, especially about the nuclei, show as irregular black smudges (Fig. 2), so dense that individual morphology can be made out only in occasional ones at the outer edges of the masses. These, together with the few scattered mitochondria within the clear zone of cytoplasm, show that the rods are plumper than in the control; a few are elongated and filamentous (some cells show filaments reaching from the nuclear

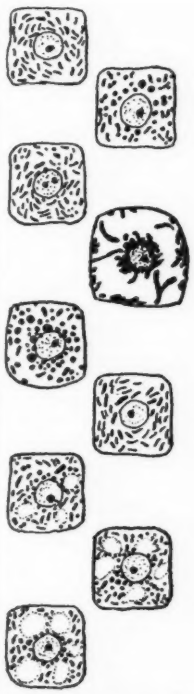
mass to the cell boundary); others are diplo- or coccoid in form. No differences of any note are observed between cells in the central and peripheral portions of the lobules.

In our carrot-feeding experiments [7] conducted a year ago the same swollen, clear cells with vacuolated cytoplasm were observed

TABLE I

Experiment 1. Effects of Carrot-Feeding on Liver Glycogen and Mitochondria

Rabbit No.	Diet	Conditions	Body weight	Liver weight	Liver glycogen	Histological glycogen	Mitochondria
			gm.	*gm.*	*per cent*		
A1	Alfalfa	No food withdrawal	1670	52	2.12	++	
A2	"	12 hrs. after food	1740	36	0.12	sl. tr.	
A3	"	24 hrs. after food	2080	47	0.26	tr.	
B1	Dried carrots	No food withdrawal	1860	58	13.10	++++...+	
B2	"	12 hrs. after food	2030	76	3.96	++++	
B3	"	24 hrs. after food	2030	50	0.55	+ (–)	
C1	Fresh carrots	No food withdrawal	2095	55	2.47	++	
C2	"	12 hrs. after food	1960	58	3.77	+++	
C3	"	24 hrs. after food	2240	65	1.08	+	



in the livers of these rabbits. At that time Best's carmine stain revealed huge quantities of glycogen and the "vacuoles" in the cells were found to correspond closely to the coarse clumps of glycogen seen in the Best's carmine preparations.

A second (B_1) animal fed on dried carrots in the same manner as the first gave similar results. The glycogen content of the liver reached 11.6 per cent, while the Best's carmine preparation showed every liver cell loaded with coarse clumps of glycogen. The mitochondrial stains by the iron hematoxylin and acid fuchsin-methyl green methods reveal almost identically the same morphology and paranuclear condensation of mitochondria as have been described.

After one of the carrot-fed animals (B_2) had fasted for 12 hours the chemical glycogen was reduced to 3.96 per cent. Glycogen is still abundant in the Best's carmine preparations, but some of the cells in the peripheries of the lobules are slightly pale. The liver cells are not so uniformly loaded as in B_1 and the granules not quite so coarse. Several counts indicate an approximate average of 35–50 granules per cell.

The mitochondria, as may be seen in the diagram, are quite uniformly distributed in the cytoplasm, in some cells moderately more abundant about the nucleus, while nearly all have assumed a coarsely granular or coccoid form. Many of the larger spheres are so perfectly round they give the impression of having a fluid or semi-fluid content.

A third animal of the group fed dried carrot (B_3) after fasting 24 hours, showed a liver nearly depleted of glycogen (0.55 per cent). Histologically only occasional small groups of liver cells contain red granules. Stains for mitochondria show a distribution and morphology indistinguishable from the control (A_1).

Two additional animals fed on a dried carrot diet, treated as the B_2 and B_3 animals respectively, gave similar results.

A group of three rabbits was fed on fresh carrots and alfalfa for a period of 12 days. These animals were treated in the same way as the foregoing group fed on dried carrots. The results may be seen in the table. Due, apparently, to the great amounts of water and roughage in the fresh carrots, rabbits are unable to consume sufficient bulk to raise the glycogen content of their livers to any marked degree. Rabbit C_1 produced 2.47 per cent glycogen in the liver, while C_2 showed 3.77 per cent after a 12 hour fast. This increase may be accounted for on the basis of the slowness of digestion of bulky food such as fresh carrots, and on individual differences between the two animals. After a 24 hour fast Rabbit C_3 had only 1.08 per cent glycogen in the liver.

TABLE II

Experiment 2. Effects of Food Withdrawal and Glucose Feeding on the Liver Glycogen and Mitochondria

Rabbit No.	Conditions	Body weight	Liver weight	Liver glycogen	Histological glycogen	Mitochondria‡
		gm.	*gm.*	*per cent*		
A1	No withdrawal of food	3210	95	1.59	+	
A2	No withdrawal of food	3030	69	0.73	+ (–)	
B1	24 hrs. without food	1890	62	0.50	+ (–)	
B2	24 hrs. without food	1960	61	0.78	+ (–)	
C1	As B, then 5 hrs. at 0°C	2740	72	0.75	+ (–)	
C2	As B, then 4 hrs. at 0°C	1990	50	0.70	+ (–)	
D1	As A, then 15 gm. glucose and killed in 3 hrs.	2970	80	2.70	++	
D2	As A, then 15 gm. glucose and killed in 3 hrs.	3430	104	5.70	++	
E1	As B, then 15 gm. glucose and killed in 3 hrs.	1760	72	4.63	+++	
E2	As B, then 15 gm. glucose and killed in 3 hrs.	1760	48	3.82	+++	
F1	As B, then 15 gm. glucose at 0 and 3 hrs. and killed 6 hrs. after first dose.	2320	96	3.50	+++	
F2		2440	91	4.17	+++	

The mitochondria show no marked changes in the cells of these livers. Quite a few granules or coccoid forms are intermixed with the rods, the former being predominant. Clear, rounded spaces in the cytoplasm of the cells in Rabbits C2 and C3 are probably fat globules. These are present in the Best's carmine preparations, as well as in the sections stained by the iron hematoxylin method.

The graph, Text-Fig. 1, shows at a glance the changes in glycogen content of the various livers of the rabbits in Experiment 1, together with the relative rapidity of the glycogenolysis. The arrows directed away from the base line indicate glycogen synthesis, while those pointing toward the base line signify glycogenolysis.

Experiment 2 (Table II): This experiment was designed to demonstrate the various effects of food withdrawal and glucose feeding on hepatic glycogen and mitochondria. The two control animals, A1 and A2, showed some variation in the glycogen content of the livers, but the mitochondria are practically identical with those seen in the control of Experiment 1. Animals B1 and B2 following a fast of 24 hours show little change from the controls. To our surprise, 4 hours at 0° C. following a 24 hour fast failed to reduce the hepatic glycogen of Rabbits C1 and C2 below that of the two previous animals. The mitochondria show only some increase in the relative numbers of granular forms.

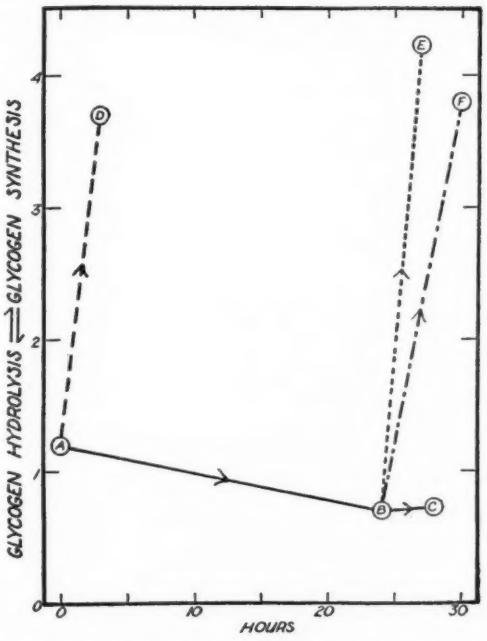
Glucose fed by stomach tube in animals D1, D2, E1, F1, and F2 brought about rapid storage of glycogen in the liver. Whether the animals are first fasted for 24 hours as in E1 and E2 makes no difference in the final result. There is some discrepancy between the figure of 5.70 per cent glycogen in Rabbit D2 and only ++ glycogen as determined by the histological method. The latter determination checks with that of the D1 liver, while the chemical determination is almost twice as great. We are unable to explain this discrepancy.

The mitochondria in the glucose-fed animals appear somewhat plumper than in the foregoing, with a greater tendency to assume the spherical form. Figure 3 represents a camera lucida drawing of three cells from F1 drawn under the same magnification as Figures 1 and 2. It may be seen that mitochondria are accumulated quite definitely about the nuclei and somewhat on the limiting cell membrane as well. Nearly all are rather coarse spherules, some of them larger than in Figure 2. Numerous clear spaces in the cytoplasm are probably the spaces occupied by fat globules.

The graph, Text-Fig. 2, which for obvious reasons is not drawn to the same scale as Text-Fig. 1, shows, in the main, rapid synthesis of glycogen.

Experiment 3 (Table III): This experiment was designed to show the effects of food withdrawal plus adrenalin on liver glycogen and mitochondria. Rabbits were fasted for 24 hours and then given

1 mg. of adrenalin intravenously, except the first two animals A1 and A2 which served as controls. Sets of two animals were sacrificed after 1, 2 and 3 hours respectively. This experiment shows that adrenalin injections after fasting first deplete the liver of glycogen during the first and second hours. By the end of 3 hours, however,



TEXT-FIG. 2. Graph (Experiment 2) showing changes in percentage of hepatic glycogen plotted against time in hours. Each letter represents the average figure for two animals. Text-Figs. 1 and 2 are drawn to different scales.

the hepatic glycogen has again returned to a normal figure (1.44 per cent).

Mitochondria may undergo, according to Cowdry, three kinds of changes, namely, qualitative, quantitative, and changes in distribution. Kater [5] found in his experiments with cats a tendency for the rods and filaments in the liver cells to take the form of granules and spherules when the glucose-glycogen equilibrium was disturbed. As we have stated before, he was dealing with very small quantities of glycogen in the individual liver cells.

Our experiments show minimal changes in the mitochondria in Experiment 3, where the quantities of glycogen are small. This experiment duplicates in part Kater's experiments with adrenalin, except that we have used rabbits instead of cats.

TABLE III

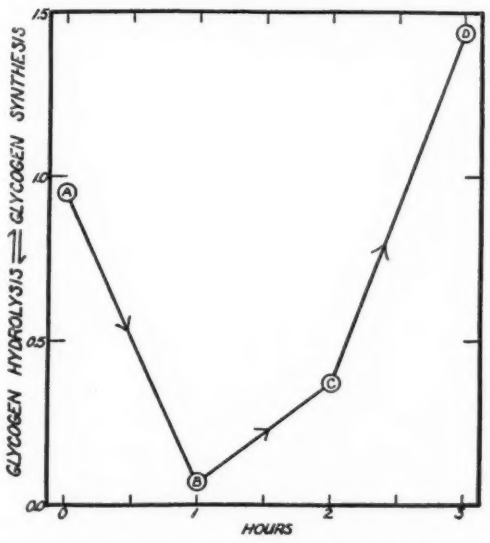
Experiment 3. Effects of Food Withdrawal Plus Adrenalin on Liver Glycogen and Mitochondria

Rabbit No.	Time after injection of adrenalin	Body weight	Liver weight	Liver glycogen	Histological glycogen	Mitochondria
		gm.	*gm.*	*per cent*		
A1	No adrenalin (control)	1350	42	0.97	(+ −)	
A2	No adrenalin (control)	1690	48	0.92	+	
B1	1 hour	1450	41	0.09	tr.	
B2	1 hour	1640	49	0.12	tr.	
C1	2 hours	1370	49	0.52	+ (−)	
C2	2 hours	1350	51	0.19	sl. tr.	
D1	3 hours	1580	44	1.44	+	

During the progress of an earlier experimental study in carrot-feeding [7] we discovered that the liver cells became loaded with glycogen beyond anything we had ever seen. It occurred to us that this would be an ideal condition in which to study mitochondria in relation to the glucose-glycogen equilibrium. If the chondriosomes take any part in this process it might be expected that relatively greater changes would be observed in them while the cell is under the strain of storing an abnormally high content of glycogen. Our

hopes have been fully realized, as can be seen by the bizarre changes wrought in the mitochondria of Rabbit B1 (Experiment 1), Figure 2.

Our experiments show a marked disturbance in the mitochondria of the hepatic cell during excessive glycogen storage and during subsequent hydrolysis. The most outstanding change is due to their redistribution within the cell, especially their condensation about the nucleus. Changes in morphology consist in hypertrophy,



TEXT-FIG. 3. Graph (Experiment 3) showing changes in percentage of hepatic glycogen plotted against time in hours. A, B and C each represents the average for two animals, D for one animal only. Scale not the same as in Text-Figs. 1 and 2.

filament-production and enspherulation. Large granules which appear to be semifluid are evident during rapid glycogenolysis.

The mitochondria show a moderate tendency to form fairly coarse spherules. In the liver of Rabbit D1 a condition is reached approaching that of F1, Experiment 2 (Fig. 3), rather closely.

The glycogen lysis and synthesis curves of Experiment 3 are illustrated in Text-Fig. 3. Here again the scale is not the same as in Text-Figs. 1 or 2.

Comment

Several investigators (Bang and Sjövall,[2] and Noël [3]) have claimed that no demonstrable relation exists between glycogen deposition and changes in the mitochondria. Altmann [8] as early as 1889 observed that the granules seen in the hyaloplasm varied in size and in disposition with the particular stage of digestion. Mann [4] states that under normal physiological activity changes in shape are usual. Imbibition of known substances from the cytoplasm by the mitochondria is well established but uncertainty exists as to what processes are involved. He further states that actual absorption probably occurs, accompanied by chemical dissociation and synthesis.

Since it is established that mitochondria of the stomach, pancreas and liver change their shape during digestion it would seem to indicate that they play some part in this important process. Cowdry [9] states in his review that the morphology of mitochondria in these organs is alike in nearly related animals. He believes that "this constancy in shape where function is similar indicates that the morphology of mitochondria is a fundamental property ingrained in the organization of the cell and that it is not always a passing trivial affair which varies from moment to moment." He further states that changes in shape of mitochondria constitute by far the most delicate criterion of many types of cell injury at our disposal.

It is not improbable that the peculiar paranuclear distribution of mitochondria at the height of glycogen storage in Rabbit B1 may be largely a mechanical effect — the mitochondria being pushed aside by the formation of coarse clumps of glycogen within the cytoplasm. The consistency of the nuclear accumulations, whether the nucleus is in the center or at one side or one end of the cell, suggests that the process is not wholly mechanical. Some chemotaxis appears to exist between nucleus and mitochondria. Uniform transformation of filamentous and rod-like mitochondria into coarse spherules during rapid glycogenolysis (Rabbit B2, Experiment 1) can be accounted for since this is a usual type of transformation observed in the mitochondria of various species of animals and plants. On the purely physical side it would appear that this process of hypertrophy and enspherulation may be the result of imbibition of fluid at the time that hydrolysis of glycogen is very active. Noël [3] speaks of the "granules of coque" which are mitochondria containing fat globules.

Recently, Kater and Smith [10] have shown that fat globules develop within mitochondria of the hepatic cell in rats following the feeding of cane sugar. The observations of these authors indicate that actual synthesis of fat takes place within the mitochondria.

Summary and Conclusions

1. Disturbances of the glucose-glycogen equilibrium in the livers of rabbits have been produced by feeding large quantities of dried and fresh carrots followed by periods of fasting; by feeding glucose to fasting animals; and by injections of adrenalin in fasting animals.

2. Excessive amounts of glycogen, as high as 13.1 per cent, were obtained in the animals fed on dried carrots. Amounts ranging from 3.5 to 5.7 per cent were obtained in the animals fed fresh carrots and in those given glucose. Injections of adrenalin produced mainly glycogenolysis.

3. Marked changes in the mitochondria were found in the animals fed on dried carrots. In place of the usual short bacilliform rods, long filaments, coarse spherules and plump rods were found condensed about the nucleus and to a lesser degree about the cell membrane.

4. Twelve hours fasting in this group produced coarse spherules without definite arrangement in the cytoplasm. Many of these appear to be semifluid.

5. Administration of glucose caused hypertrophy and enspherulation of mitochondria with some tendency to paranuclear arrangement.

6. We conclude that some relation exists between the mitochondria of the hepatic cell and the glucose-glycogen equilibrium. Whether or not the chondriosomes act as catalysts, as they appear to do in the synthesis of fat within the hepatic cell, we are unable to say.

REFERENCES

1. Arnold, Julius. Zur Morphologie des Knorpelglykogens und zur Struktur der Knorpelzellen. *Virchows Arch. f. path. Anat.*, 1908, **194**, 266–286.

2. Bang, I., and Sjövall, E. Studien über Chondriosomen unter normalen und pathologischen Bedingungen. *Beitr. z. path. Anat. u. z. allg. Pathol.*, 1916, **62**, 1–70.

3. Noël, Robert. Recherches histo-physiologiques sur la cellule hépatique des mammifères. *Arch. d'anat. micr.*, 1923, **19**, 1–156.

4. Mann, Frank C. The Cytology of the Liver and its Functional Significance. Special Cytology, edited by E. V. Cowdry. Paul B. Hoeber, Inc., New York, 1928, **1**, 205.

5. Kater, J. McA. Variations in the mitochondria of the hepatic cell in relation to alterations of the glycogen-glucose equilibrium. *Anat. Record*, 1931, **49**, 277–288.

6. Clark, J. A., and Hair, G. E. Observations on the mitochondria of the hepatic cell of the frog in normal and hyperglycemic states. *Ztschr. f. Zellforsch. u. mikr. Anat.*, 1932, **15**, 123–130.

7. Hall, E. M., and MacKay, E. M. Experimental hepatic pigmentation and cirrhosis. II. The effect of heavy carrot-feeding on the rabbit's liver. *Am. J. Path.*, 1931, **7**, 343–350.

8. Altmann, Richard. Die Struktur des Zellkernes. *Arch. f. Anat. u. Entwck., Anat. Abt.*, 1889, 409–411.

9. Cowdry, E. V. General Cytology. University of Chicago Press, Chicago, 1924, 313.

10. Kater, J. McA., and Smith, Dorothea M. The formation of fat in the hepatic cell. *Anat. Record*, 1932, **52**, 55–68.

DESCRIPTION OF PLATE

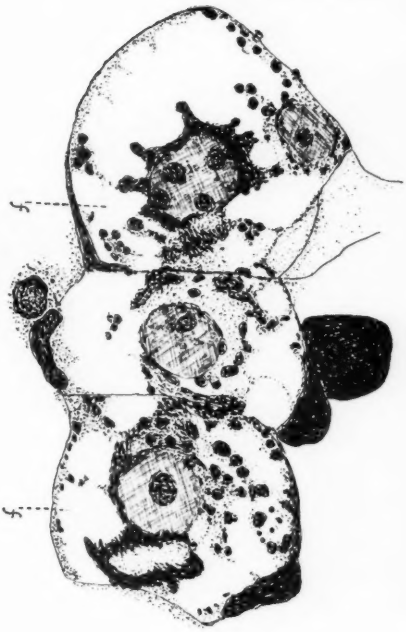
PLATE 31

FIG. 1. (Experiment 1, Rabbit A1.) Drawing of several liver cells from control, showing the usual type of rod-like mitochondria. The solid black bodies are red blood cells. (Camera lucida, Zeiss 2 mm. obj., 15 × ocular.)

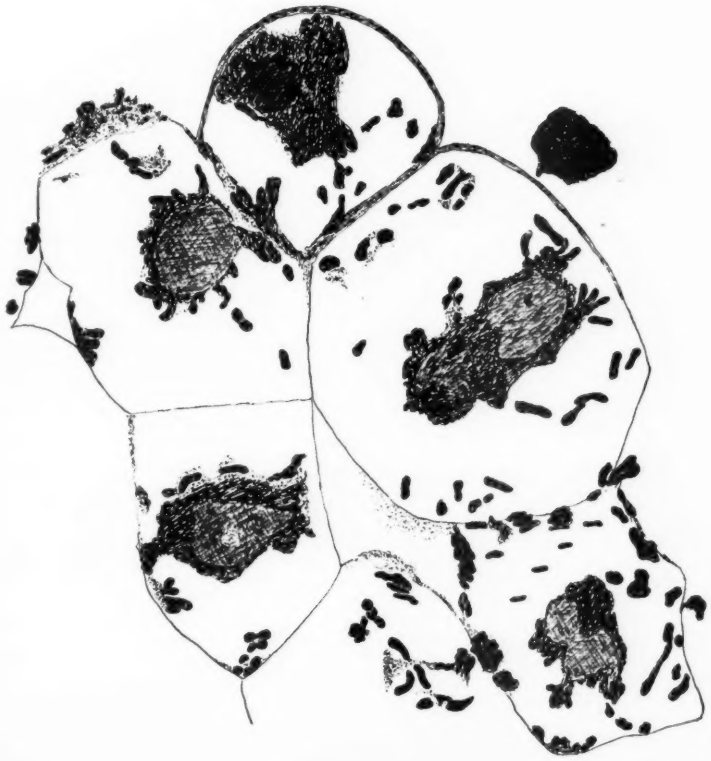
FIG. 2. (Experiment 1, Rabbit B1.) Drawing of a group of liver cells under the same magnification as Fig. 1, showing swelling of the cells, clear cytoplasm, hypertrophied and filamentous mitochondria with accumulation about the nuclei.

FIG. 3. (Experiment 2, Rabbit F1.) Drawing of three liver cells under the same magnification as Figs. 1 and 2, showing hypertrophy and enspherulation of mitochondria with tendency to accumulate about the nuclei. Clear spaces (f) in the cytoplasm are apparently due to fatty droplets.

1



3



2

Hall and MacKay

Mitochondria and Glucose-Glycogen Equilibrium

THE SIMILARITY OF VIRUS PNEUMONIA IN ANIMALS TO EPIDEMIC INFLUENZA AND INTERSTITIAL BRONCHO-PNEUMONIA IN MAN *

HOWARD ANDERSON MCCORDOCK, M.D., AND RALPH S. MUCKENFUSS, M.D.

(From the Department of Pathology and the Department of Internal Medicine, Washington University School of Medicine, St. Louis, Mo.)

A characteristic and unique type of pneumonia frequently complicates certain epidemic diseases. This condition which MacCallum [1] has styled interstitial bronchopneumonia is observed most frequently in fatal cases of measles, whooping cough and epidemic influenza. During an influenza epidemic or following an outburst of measles the pathologist suddenly encounters many such cases, but even under ordinary circumstances in every large hospital with a pediatric service he is kept aware of the condition by deaths from whooping cough and measles. Although the pathological anatomy of interstitial bronchopneumonia was described clearly many years ago by Bartels [2] in Germany, and by Delafield [3] in this country, it has become increasingly familiar since the World War through the publications of MacCallum,[4] who studied the pneumonias associated with the epidemics of measles in the United States Army camps, and those of Opie and his associates,[5] who concerned themselves with postinfluenzal pneumonia.

The gross lesions of interstitial bronchopneumonia can be observed best on a smoothly cut surface of the lung, which is often studded with small nodules of grayish yellow tissue that sheath the branching bronchioles. A thick exudate usually can be squeezed from the central bronchioles, which generally show thickened walls and stand out from the surface as rigid tubes. These yellow peribronchiolar nodules have a striking gross resemblance to tubercles or foci of tuberculous bronchopneumonia, as pointed out long ago by Honl.[6] Although usually small, they may develop large enough to become confluent, but even when they coalesce the presence of the thickened bronchial walls, often pink due to granulation tissue, suffices to prevent one from confusing the consolidated areas with other types of pneumonia. The intervening lung tissue is usually

* Received for publication September 26, 1932.

collapsed and often dark red, but it is not apt to show hemorrhage or edema, except in individuals dying at the height of an influenza epidemic.

Under the microscope the central bronchioles are plugged with granular leukocytes, between and sometimes within which bacteria of various types are found. The most striking feature, however, is the great thickening of the bronchial wall and of the alveolar walls in an encircling zone of lung tissue. This thickening is due mostly to an infiltration with large mononuclear cells, lymphocytes, plasma cells and proliferating fibroblasts. The consolidated alveoli are often filled exclusively with large mononuclear cells, but in places they are occupied by tangled masses of dense, red-staining fibrin in which there is an occasional wandering cell. Of course, many alveoli contain polymorphonuclear leukocytes as well as red blood corpuscles, and this is especially true in material from the influenza epidemic of 1918 in which the presence of a variety of bacteria complicates the picture. In many cases of measles or whooping cough, however, the polymorphonuclear cells are scarce and often confined to the bronchioles. The lymphatics of the septa and bronchial walls, together with those about the blood vessels, are dilated and filled with inflammatory cells mixed perhaps with fibrin and, at times, bacteria. These are briefly the important characteristics of interstitial bronchopneumonia that distinguish it from those of the ordinary broncho- or lobular pneumonia,* which so frequently develop terminally in all types of illnesses, and in which some bronchioles and their connected alveoli are filled with a uniform exudate made up of polymorphonuclear leukocytes, fibrin, red cells and bacteria without the cellular infiltration of the interstitial tissue.

The conclusions we have reached have been based upon the study of autopsy material and upon the results of experimental infection in animals. We have examined the sections from 50 autopsies upon patients who died of epidemic influenza during the 1918–19 pan-

* The terms bronchopneumonia and lobular pneumonia are used interchangeably to designate a patchy consolidation of the lung. Each term leaves much to be desired since all acute pneumonias involve the bronchi, and the areas of consolidation referred to are never strictly lobular. We shall use the term lobular pneumonia except when the patchy consolidation is accompanied by a cellular infiltration of the interstitial tissue, in which case MacCallum's recently designated term "interstitial bronchopneumonia" will be employed.

demic. In addition we have studied 25 autopsied cases of measles, 40 of whooping cough and 100 others as controls. The majority of the latter were from children who had died of diseases other than those forming the basis of this report.

Experimental Procedures

While studying vaccine virus pneumonia in rabbits [7] we were impressed by the similarity of the lesions to those found in interstitial pneumonia as well as in the hemorrhagic and edematous type of consolidation observed in the lungs of individuals dying a few days after the onset of epidemic influenza. In this work bacteriologically sterile vaccine virus (neurovirus of Levaditi) was injected into the lungs through the trachea, and the animals were killed from 1 to 5 days later. In the majority of the animals no bacteria could be demonstrated in the lungs. However, two animals not included in our published report contracted a pulmonary bacterial infection which complicated the experimentally produced virus pneumonia. Both of these animals developed a pleurisy with a thin, turbid brown fluid. Cultures showed the presence of a streptococcus in pure culture in one animal, and mixed with staphylococci and colon bacilli in the other. The lesions in these two animals so closely resembled the changes found in certain cases of epidemic influenza that the experimental use of a combination of virus and bacteria was at once suggested. The interval between the injection of bacteria and virus was varied in different series of experiments. In one series bacteria were mixed with the virus before inoculation and the mixture injected into the trachea. In other experiments diluted virus was injected first and followed from 1 to 7 days later, in different series, by the injection of a suspension of bacteria. Animals were killed at intervals varying from 1 to 18 days after the first inoculation of virus.

The virus was prepared by diluting with Locke's solution an emulsified rabbit testicle 4 days after intratesticular inoculation with the neurovirus of Levaditi. In experiments in which virus alone was used 1 to 2 cc. of a 1:10 dilution were injected. When bacteria were also injected a 1:10 dilution proved too strong, so that 1 to 2 cc. of a 1:100 dilution were usually employed in the latter experiments.

Three types of bacteria were used. One was a hemolytic streptococcus isolated from a spontaneous infection in a rabbit. Another was a staphylococcus cultured from the lung of a child with post-pertussis interstitial bronchopneumonia, and the third organism was a stock strain of *B. pertussis*. These organisms were grown on blood agar slants from which thin suspensions were prepared in salt solution.

Individual protocols will not be given, for over 100 animals have been used in these studies and the crucial experiments have been repeated often enough with sufficient controls to eliminate fortuitous reactions.

Vaccine Virus Pneumonia

In another place [7] we have described in detail the pulmonary changes induced by vaccine virus without bacteria, and here only the more important lesions will be discussed. Twenty-four hours after the injection of virus there is a marked tracheitis and bronchitis with a red, swollen mucosa. The lungs show elevated patches of translucent gelatinous consolidation, some of which become hemorrhagic after the second day, producing firm, dark red to purple-colored areas. About the third or fourth day, on a cut surface of the lung in an involved area the bronchi are prominent due to thickening of the walls. In an occasional animal this thickening and infiltration extend beyond the bronchioles to produce small yellowish peribronchiolar nodules resembling tubercles, which are very conspicuous where the lung tissue forming the background is hemorrhagic. The bronchial lymph nodes are enlarged and soft.

Microscopic study shows the areas of gelatinous consolidation, the earliest gross lesion, to consist of groups of alveoli filled with coagulated edematous fluid and varying amounts of fibrin. At this stage the alveolar walls show no structural change, although their capillaries are at times congested. Mononuclear cells may escape into the alveoli in small numbers. In later stages this lesion may resolve or it may progress in two different ways. In the most acute reactions the alveolar epithelium degenerates and desquamates. Damage to the capillaries causes hemorrhage into the alveoli. As this lesion develops the entire alveolar wall, including the capillary, degenerates and in this way large irregular areas of lung tissue, although retaining their structural outline, become completely ne-

crotic, resembling the center of an infarct. This necrosis appears to be due to the direct action of the virus upon the tissue and is not the result of thrombosis of blood vessels. If the animal survives great numbers of polymorphonuclear leukocytes invade the necrotic tissue. We suggest that this acute process be called "hemorrhagic virus pneumonia." Instead of this hemorrhagic and necrotizing process a proliferative cellular lesion may develop. When this takes place the bronchioles and the alveolar walls become thickened by an infiltration with mononuclear cells. The infiltration is most marked in the alveoli close to a bronchiole or in the neighborhood of a blood vessel, the adventitia of which is thickened and infiltrated. The cells of the alveolar epithelium enlarge and the great frequency of mitotic figures clearly indicates their active multiplication. These large mononuclear cells become more numerous in the alveoli and in some they displace all other elements. It is proposed that this proliferative reaction be termed "interstitial virus pneumonia." Although these two lesions are almost always distinct and separate, nevertheless, elements of each can occasionally be recognized in one lesion.

These two types of lesion are not regarded as different stages of the same process but rather as quantitative reactions to different concentrations of virus. A large quantity of strong virus injected into the lungs of a normal animal tends always to produce hemorrhagic virus pneumonia with extensive necrosis, a minimal cellular proliferation and early death. A small quantity of it, or a more dilute virus, as a rule calls forth the proliferative cellular lesions of interstitial virus pneumonia, which is not immediately fatal. A moderate amount of virus introduced into the lungs of a vaccine-immune animal, or the injection of virus mixed with immune serum into a normal animal, results in a proliferative lesion, if there is any reaction at all.

The mucosa of the larger bronchi is hyperemic, but the epithelial cells are usually not destroyed. In the bronchioles of an involved area, however, a patch of necrotic epithelium is frequently seen with collections of polymorphonuclear leukocytes about the dead cells. Cytoplasmic inclusions (Guarnieri bodies) are found in the bronchial epithelium in about 10 per cent of non-immune animals.

The perivascular lymphatics, as well as the lymphatics of the septa and those of the bronchial walls, become greatly distended and

often filled with thrombus-like coagula of albuminous fluid and fibrin, in the meshes of which large mononuclear wandering cells, leukocytes and lymphocytes are entangled. The walls of the adjacent blood vessels become edematous and the connective tissue and muscle fibers are pushed apart. Foci of necrosis appear in the adventitia and may extend into the media. When this occurs large numbers of polymorphonuclear leukocytes and a few mononuclear cells infiltrate the vessel wall.

It must be emphasized that all these lesions occurred in the absence of bacteria. Repeated cultures of lung tissue remained sterile, and bacteria were never found in the many sections of the most severe lesions stained by various methods for the demonstration of bacteria in tissues.

Virus Pneumonia with Superimposed Bacterial Infection

With the exception of typical lobar pneumonia, which is notoriously difficult to induce experimentally, we have been able to reproduce by the use of vaccine virus and bacteria practically every pulmonary lesion that has been described as a complication of influenza or measles. The effect of the bacteria alone was first studied. When injected into the lungs through the trachea the animals responded differently to each of the three strains of bacteria used. The only reaction noticed after injection of large quantities of *B. pertussis* was a mild inflammation of the tracheal and bronchial mucosa. Pneumonia was never observed. Because of its apparent non-virulent character this old stock strain of the organism was used in only a few experiments after vaccine virus and in each instance the virus pneumonia was not altered in any remarkable manner.

The streptococcus isolated from a rabbit regularly produced an ordinary lobular pneumonia with a typical exudate of polymorphonuclear leukocytes in bronchioles and a group of connecting alveoli. Even in large numbers this organism seldom proved fatal. The staphylococcus recovered from the lungs of a child dying of pertussis proved to be the most virulent organism. Small numbers of this coccus incited a lobular pneumonia, but a larger number (one-half of an agar slant) produced death in 24 to 48 hours from septicemia. It was with these last two mentioned organisms that most

of the experiments with combined injections of virus and bacteria were carried out.

It will be recalled that vaccine virus alone produces two distinct types of pneumonia. One is an acute hemorrhagic and edematous consolidation with foci of necrosis, and the other an interstitial cellular infiltration with large mononuclear cells, fluid and fibrin in some of the alveoli.

The important lesions or complications superimposed on virus pneumonia by the simultaneous or subsequent introduction of bacteria may be classified as follows: (*a*) acute bronchitis and lobular or bronchopneumonia; (*b*) abscess formation; (*c*) pleurisy and empyema; and (*d*) bronchiectasis, squamous cell "metaplasia" of bronchial epithelium and organizing pneumonia.

Acute Bronchitis: It has already been pointed out that the characteristic cellular exudate of pure vaccine virus pneumonia is composed principally of large mononuclear wandering cells and that polymorphonuclear leukocytes appear in large numbers only after the virus has caused necrosis of the exudate or of the pulmonary tissue when the granular leukocytes infiltrate the dead tissue. Within 24 hours after the introduction of bacteria into the lungs of an animal, which already has a virus pneumonia, a typical pyogenic exudate composed of polymorphonuclear leukocytes, bacteria and a scant amount of fibrin appears in the terminal bronchioles and in a varying number of connected alveoli. In some cases the bacteria are found only in the bronchioles and do not reach the alveoli. Frequently the bacteria reach parts of the lung that have not previously been affected by the virus, and in such places there is a pure lobular pneumonia.

Abscess Formation: Pulmonary abscesses, both solitary and multiple, occurred in many of the animals that survived the injection of bacteria for a week or 10 days. The abscesses were most frequently situated in the lower lobes posteriorly and were often close to the pleural surface. Some of the abscesses had an opaque, soft yellow center occasionally surrounded by a narrow zone of hemorrhage; others had a softened center and an ill-defined edge that fused with a flabby, consolidated peripheral zone of lung tissue, with or without hemorrhage. Microscopically some of these irregular abscesses appear to have been produced by the bacteria spreading rapidly from the central bronchiole throughout an area that already

had been rendered necrotic by the action of virus. The center of such an abscess is a formless mass of necrotic tissue and bacteria, but toward the periphery there is often a zone of necrotic lung tissue resembling in every respect the necrosis observed in pure virus pneumonia. Bacterial stains seldom reveal any bacteria in the outer part of this necrotic edge. If one is unfamiliar with virus pneumonia it would be natural to ascribe the peripheral necrotic zone to the diffusion of bacterial toxins into the surrounding lung tissue from the center of the abscess. Of course all the abscesses may originally have started in damaged tissue, for it is well known that lung abscesses cannot readily be produced by injecting ordinary pyogenic bacteria into a normal lung. However, it seems likely that in some instances an abscess started at the center of a large area of virus necrosis and the animal died before the bacterial infection involved the entire necrotic area.

Pleurisy and Empyema: A serofibrinous pleurisy was found in two animals that contracted a spontaneous bacterial infection after the injection of vaccine virus. In one animal the pleurisy was bilateral and in the other the right side only was affected. The fluid was thin and light brown in color with numerous shreds and flakes of fibrin that settled to the bottom. A streptococcus was cultured from this fluid. Microscopically these lungs show a widespread hemorrhagic virus pneumonia with patches of necrosis that involve practically the entire lower lobes. In addition there are areas of lobular pneumonia, and where these extend to the pleural surface it is covered with a layer of fibrin. Streptococci can be found everywhere in the necrotic lung tissue, usually without any reaction about them. There are no abscesses.

Patches of fibrinous exudate were often encountered upon the pleura of animals that developed lobular pneumonia after the injection of bacteria wherever the areas of consolidation reached the surface of the lung. Occasionally, when such patches were numerous a few cc. of cloudy or thick yellow fluid were found in the pleural cavity. Therefore no sharp distinction could be drawn between serofibrinous and purulent pleurisy.

Empyema has occurred three times in animals surviving the injection of bacteria for 7 to 10 days. The only essential difference between the three cases concerned the amount of pus in the pleural cavities. In every instance the empyema was accompanied by a

subpleural abscess that had ruptured into the pleural cavity. The animal killed 10 days after the injection of bacteria had an abscess in the upper part of the right lower lobe, which had ruptured into the fissure, thus causing an interlobar empyema. Thick, creamy yellow pus extended through the fissure and out into the pleural cavity posteriorly where there was a large encapsulated empyema pocket extending throughout the entire length of the thoracic cavity. Adhesions between lung and chest wall along the axillary line prevented the pus from reaching the anterior portion of the pleural cavity.

Microscopically the lungs show a well advanced virus pneumonia with marked cellular infiltration of the interstitial tissue. Many of the alveoli contain mononuclear cells and dense masses of fibrin. There is also a superimposed purulent bronchitis and a lobular or bronchopneumonia with abscess formation.

In pure virus pneumonia the lymphatics of an involved area are widely distended with albuminous fluid, fibrin, and in places with groups of inflammatory cells, principally large mononuclear wandering cells. Many of the animals that developed pleurisy and empyema had masses of bacteria in these dilated lymphatics. In some sections the subpleural lymphatics contained many organisms. Although the granulocytes appeared to be more numerous in these infected lymphatics than in the same channels in pure virus pneumonia, they were not so abundant as might be expected in the presence of so many bacteria. It is obvious that bacteria can spread more rapidly throughout a lung that already has a well developed virus pneumonia, by way of the dilated lymphatics, than they can through a normal lung in which the lymphatic channels have not previously been altered. In the control animals, which had received only bacteria, this rapid dissemination by way of lymphatics was never observed. Thus it appears that a virus pneumonia induces such changes in the lung that bacteria, which in normal animals can incite only a limited infection, are enabled to multiply and spread throughout it causing extensive and fatal lesions. The same bacteria are incapable of producing such lesions without the preliminary action of the virus upon the pulmonary tissue. At least two lesions of virus pneumonia are conducive to the more rapid growth and dissemination of bacteria. The foci of pulmonary necrosis support a more luxuriant bacterial growth than normal tissue will allow, and

the wide dilatation of the lymphatic channels enhances the opportunities for the more rapid distribution of organisms throughout the lung.

Bronchiectasis and Associated Lesions: A slight dilatation of the smaller bronchi was frequently observed in animals that developed severe purulent bronchitis or abscesses. One of the animals with empyema showed marked distention of the bronchi in the lower lobes. The bronchi were uniformly distended to about twice their normal diameter, and in a few places localized, spherical bronchiectatic cavities were formed. A thick mucopurulent fluid filled the bronchial tree, and after the exudate was washed away the mucosa was found to be of a deep red color, due to hyperemia and petechial hemorrhages. Shreds of fibrin were attached to the epithelium in a few places.

The epithelial lining of the bronchiectatic cavities is in places completely destroyed; elsewhere only parts of it are necrotic and the dead tissue is usually covered with a layer of fibrin and leukocytes. Regeneration of the epithelial cells has resulted, in a few areas, in the appearance of flattened cells arranged in several layers resembling squamous epithelium. This change is identical with the condition described by many authors as squamous cell metaplasia.

In some bronchi, especially in those in which there is extensive destruction of the mucosa, inflammatory cells infiltrate the entire wall. Polymorphonuclear leukocytes are found in greatest abundance near the exposed surface, and mononuclear cells, lymphocytes and plasma cells deeper in the wall. The alveoli surrounding such bronchi usually contain masses of fibrin or a cellular exudate composed of a few mononuclear cells and many granular leukocytes. In the most chronic bronchiectatic lesions fibroblasts, and at times newly formed capillaries, can be found in the bronchial walls.

In one instance an organizing pneumonia was found in an animal with bronchiectasis. A cellular fibrous tissue replaced the exudate in a group of alveoli about dilated bronchi. This loose fibrous tissue in many places extended by means of narrow processes through openings between neighboring alveoli. Capillaries were never found in the fibrous tissue within the alveoli.

The Etiology of Epidemic Influenza

Despite the voluminous literature on influenza and the bewildering number of clinical and experimental investigations carried on during and since the 1918 pandemic, there is still no unanimity of opinion concerning its etiology. Indeed there is a difference of opinion as to what lesions constitute influenza, *per se*, and what should be regarded as complications. In general the prevailing ideas regarding the causative agent are represented by one of the two following conceptions: (*a*) influenza is due to the bacillus influenza of Pfeiffer, (*b*) the etiological agent of influenza is a filter-passing virus.

The Rôle of B. Influenzae

Many investigators still regard the bacillus of Pfeiffer as the etiological agent of influenza because it so frequently has been found somewhere in the respiratory tract of individuals ill with this disease. In one investigation at Camp Pike, Opie, Blake, Small and Rivers [5] found *B. influenzae* "invariably present in all cases of influenza." However, they discovered that in normal groups the incidence of the same organism varied between 11 and 88 per cent, and that about 80 per cent of individuals with measles also harbored this bacillus. Park [8] found it in 80 per cent of cases. Wolbach,[9] Pritchett and Stillman,[10] and Spooner, Scott and Heath [11] all found the bacillus frequently. In England, McIntosh [12] found the bacillus in 42 out of 69 cases, and other English reports also show a lower incidence of the bacillus than our own. Messerschmidt, Hundeshagen and Scheer [13] in Germany report the presence of the bacillus in 90 per cent of individuals affected during the height of the epidemic. On the other hand, the Camp Lewis Pneumonia Unit,[14] Kinsella,[15] Hirsch and McKinney,[16] as well as some investigators in England and Germany, found *B. influenzae* rather infrequently. However, in spite of these negative results there can be little doubt that *B. influenzae* is present in the majority of individuals suffering from epidemic influenza.

The influenza bacillus is frequently found in the nasopharynx of healthy individuals, although during epidemic periods its incidence increases. To cite only one example, Williams [17] and her coworkers found 40 per cent of normal persons as well as 92 per cent of those

ill with influenza harboring the bacillus. They also observed an increase in the frequency of pneumococcus and of hemolytic streptococcus as well as *B. influenzae* in patients suffering from influenza.

B. Influenzae is found associated with a variety of other conditions. Mention has already been made of its presence in 80 per cent of patients with measles at Camp Pike. These cases occurred during the influenza epidemic, but Wollstein,[18] Davis,[19] Liebscher[20] and others have found it associated with measles during interepidemic periods, and Boggs[21] and Lord[22] report its frequency in a variety of acute and chronic diseases of the respiratory tract. The bacillus has also frequently been isolated from children suffering from whooping cough.

Opinions concerning the etiological rôle of the influenza bacillus are as divergent as the above reports of its incidence in the disease. The Medical Research Committee of Great Britain[23] were of the opinion that the orthodox conception that Pfeiffer's bacillus is the cause of epidemic influenza is no longer tenable. MacCallum[24] and Kinsella[15] regarded the organism as a secondary invader. MacCallum, however, thought that *B. influenzae* was the cause of a purulent bronchitis and a lobular or bronchopneumonia complicating some cases of influenza. Opie *et al*[5] say: "The constant association of *B. influenzae* with influenza suggests that it is the cause of the disease." He adds, however: "It is possible that *B. influenzae* is a secondary invader, entering the respiratory tract when susceptibility is increased by an unknown virus causing influenza."

In order to reconcile the prevalence of *B. influenzae* in the nasopharynx of healthy individuals, with the view that this bacillus is the cause of influenza, those who champion this conception claim that *B. influenzae* suddenly undergoes a rapid increase in virulence, changing from a harmless saprophyte to a highly virulent pathogenic organism. In spite of the well known fact that the virulence of an organism can be enhanced by repeated passages from host to host, such an extreme increase in virulence as would be necessary to uphold this view finds no support in our knowledge of the increase and decrease of bacterial virulence in epidemic diseases caused by microörganisms.

Of all the experimental efforts to reproduce influenza with cultures of *B. influenzae* those of Opie and coworkers,[5] performed at Camp Pike during the epidemic in the fall of 1918, were done under

especially favorable circumstances. Suspensions of recently isolated cultures were used. Although these caused a mild, self-limited respiratory illness, somewhat similar to slight attacks of influenza in man, it required the addition of virulent streptococci or pneumococci to provoke severe pulmonary lesions; but these organisms are capable of producing the same changes without the assistance of the influenza bacillus.

By the use of fluid cultures of *B. influenzae* and the peritoneal exudates of infected animals, Blake and Cecil [25] were able to initiate an acute respiratory infection similar "in its clinical course, symptoms and complications with influenza." Ten out of twenty-seven animals developed a bronchopneumonia. The chief characteristics of this lesion were hemorrhage and edema, bronchiolitis and peribronchial infiltration. We believe that true interstitial bronchopneumonia was not reproduced. The predominant cells in the exudate and infiltrations appear to have been polymorphonuclear leukocytes. The hemorrhagic and edematous exudates, of course, resembled the early acute lesions of influenza in man. These changes are to be expected with liquid cultures, for in them Parker [26] proved the presence of a bacterial toxin. Fildes and McIntosh,[27] as well as Huntoon and Hannum,[28] were able to produce edema and hemorrhages in the lungs and elsewhere with filtrates of fluid cultures of *B. influenzae*. Winternitz and coworkers [29] have shown that similar hemorrhagic and edematous lesions with necrosis of the alveolar epithelium occur after exposure to toxic gases such as chlorine and phosgene, and comment upon their similarity to the lesions found in the acute hemorrhagic stage of influenza in man. Although it seems possible to produce a self-limited respiratory affection somewhat similar to mild cases of human influenza with massive cultures of *B. influenzae*, or even to incite a lobular pneumonia, and with fluid cultures or their filtered toxins to effect hemorrhages and edema in the lungs of animals, no one has yet produced pulmonary changes with this bacillus that simulate those of epidemic influenza in man, or its more important complications. Any adequate explanation of the pathogenesis of influenza must not only consider the acute phases of the disease but must also account for the interstitial bronchopneumonia, as well as for the variety of pulmonary complications that frequently follow.

Evidence for a Filterable Virus

A great deal of experimental work, especially since 1918, supports the conception that influenza is caused by a filter-passing virus. During the second wave of the late pandemic, evidence was brought forth to support this idea by Gibson, Bowman and Connor [30] in England, and by Nicolle and Lebailly [31] of the French army. In 1919 Yamanouchi, Sakakami and Iwashima,[32] using filtered and unfiltered samples of sputum from influenza patients, reproduced clinical influenza in 18 out of 24 volunteers by injections into the nose and throat. The remaining 6 had recovered from the spontaneous disease and were therefore immune. Filtered blood produced similar results, but pure cultures of *B. influenzae*, or mixtures of this and other bacteria found in the respiratory tract, failed to induce illness in 14 healthy persons.

All these experiments merely prove the existence of a toxic substance in the sputum of patients suffering from influenza, and are open to the criticism that a living virus was not demonstrated. Passage experiments, if performed, were negative. Carefully controlled experiments have been undertaken by Lister and Taylor,[33] and by McIntosh,[12] and all have given negative results. A survey of the published work upon the relation of a filterable virus to influenza fails to reveal any convincing proof of its presence.

The Lesions of Influenza and Their Similarity to Those of Virus Pneumonia

The earliest acute pulmonary lesion of influenza is a hemorrhagic and edematous lobular consolidation. Areas resembling hemorrhagic infarcts are not uncommon. Microscopically the interlobular septa and the connective tissue about the vessels and bronchi are edematous. Groups of alveoli are filled with coagulated albuminous fluid, red blood cells or fibrin. The appearance of the alveolar contents varies. In some the edematous material is granular, in others a clear, pink-staining colloid-like mass is seen. Varying amounts of fibrin and red cells may be found in the alveolar coagulum. Occasionally dense masses of red blood cells fill a group of alveoli and make it impossible to distinguish the alveolar walls traversing the area.

Necrosis of the epithelium of the bronchioles and alveoli is frequent and in places this necrosis is not confined to the epithelial

layer but extends into the walls of bronchioles and alveoli. In this manner areas of lung tissue are killed *en masse*, producing foci of necrosis.

This early hemorrhagic lesion found a few days after the onset of the disease has been emphasized by Wolbach,[9] Winternitz *et al*,[29] and Goodpasture and Burnett.[34] The latter authors point out the scarcity of bacteria at this stage and suggest that the injury may be due to some toxic agent acting upon the lung tissue. Winternitz and coworkers remark about the similarity of the lesions to those occurring after exposure to irritating gases.

In a short time other lesions appear, usually from 5 to 10 days or later after the onset of the disease. These may be described briefly as a cellular infiltration of the interstitial tissue, a non-purulent alveolar exudate and lobular or bronchopneumonia. Taken together these constitute interstitial bronchopneumonia.* Lobar pneumonia, pleurisy, empyema, bronchiectasis, organizing pneumonia and other lesions may also follow.

The edematous and hemorrhagic consolidation with occasional foci of necrosis found in the lungs of animals after the injection of strong vaccine virus closely resembles the early lesions found in epidemic influenza. The paucity of bacteria in the latter is significant, since the experimental virus lesions are bacteriologically sterile. The only elements of interstitial bronchopneumonia found in simple virus pneumonia are the interstitial cellular infiltration, the mononuclear exudate and the fibrin masses in the alveoli. The lobular or bronchopneumonic component of interstitial bronchopneumonia, however, may be superimposed upon vaccine virus pneumonia by the intratracheal injection of bacteria that induce a purulent bronchitis and lobular pneumonia with a polymorphonuclear exudate in the bronchioles and connecting alveoli.

* The strict etymological meaning of the term "interstitial bronchopneumonia" probably indicates an interstitial inflammation or pneumonia which is bronchial in distribution, and does not include the lobular or bronchopneumonic element consisting of a polymorphonuclear exudate in the central bronchioles and in groups of adjacent alveoli that usually accompanies the interstitial inflammation. According to this strict interpretation it would be necessary to speak of interstitial and bronchopneumonia in order to include both elements. However, from MacCallum's description of the condition,[1] it is evident that he intended the term "interstitial bronchopneumonia" to connote the polymorphonuclear exudate in the bronchioles and alveoli, as well as other salient features that are not defined by the term "interstitial bronchopneumonia." It is in this broader sense that we use the term.

The more chronic pulmonary and pleural lesions that can be produced by a combination of virus and bacteria have already been described, and here it is only necessary to point out their similarity to many of the various complications that sometimes follow epidemic influenza. Lobar pneumonia is the only common complication of influenza that we have not reproduced, perhaps because the pneumococcus was not used, or because of the type of animal used.

Opie [5] and coworkers have described an acute endophlebitis that they observed in association with interstitial suppurative pneumonia and in other lesions complicating influenza. This vessel change resembles the vascular lesion we have described in vaccine virus pneumonia. Their similarity can be appreciated by comparing Figure 14 in Opie's book with Figure 5 in our paper on vaccine virus pneumonia.[7] Although there were bacteria in all the lungs in which Opie observed this endophlebitis, the presence of similar lesions in rabbit lungs free from bacteria indicates that this lesion may also be caused by a filterable virus.

A thin, ribbon-like layer of hyaline material lining alveoli and alveolar ducts is sometimes found in interstitial bronchopneumonia, whether the latter is the result of influenza, measles or whooping cough. Wolbach and Goodpasture regarded this hyaline layer as characteristic of postinfluenzal pneumonia. Although masses of dense fibrin, staining brilliantly with eosin, are commonly found in animals with virus pneumonia, this homogeneous hyaline layer lining the alveoli was observed only a few times in our experimental lesions.

In some cases of influenza complicated by a streptococcus, areas of pulmonary necrosis overgrown with this organism have been observed, and between these foci streptococci have usually been present in smaller numbers. The opinion has frequently been expressed that the necrosis in these foci is due to the localized growth of so many bacteria. Similar foci of necrosis not overgrown with bacteria can be found in the lungs of individuals dying a few days after the onset of influenza, and in our experimental animals massive colonies of bacteria were frequently found at the centers of such necrotic foci, which apparently had been previously produced by virus, while in other well preserved regions of the lung the organisms had not grown so prolifically. Because of these observations it seems probable that, in some cases of influenza at least, these

areas of necrosis may antedate the entrance of the streptococcus and the remarkable growth of the latter in these foci may be the result of the preëxisting necrosis and not its cause.

While the experiments that have been performed with vaccine virus and bacteria in rabbits cannot prove the existence of a virus in epidemic influenza in man, the similarity of the anatomical lesions suggests such a possibility.

The Etiology and Anatomical Lesions of Measles

Although most workers are now agreed that measles is caused by a filterable virus, a few still present evidence in support of various bacteria. The most suggestive work is that of Tunnicliff and Hoyne,[35] who describe a small Gram-positive, filter-passing, green-producing diplococcus as the causative agent. Tunnicliff and her associates have demonstrated antibodies for this organism in the blood of measles patients. Long and Cornwell,[36] however, did not succeed in finding organisms of this group in 26 cases of measles; and Bradford [37] as well as Smith [38] could not distinguish these cocci isolated from measles from similar hemolytic strains obtained from healthy individuals. Conclusive evidence to prove that these cocci are the cause of the disease is wanting. Typical measles has not been produced by the injection of cultures of these organisms into human beings, as is true of the production of the disease by other methods.

The work of Hektoen,[39] and of Blake and Trask,[40] supplies convincing evidence in favor of the theory that measles is due to a filterable virus. Hektoen showed that the disease can be induced in susceptible human beings by injecting the blood of measles patients taken shortly after the rash appears. Blake and Trask were able to reproduce a condition very similar to human measles in monkeys by the injection of the filtered nasal washings from patients ill with measles. In passage experiments the disease was carried through six monkeys by injecting ground-up tissue or blood. Degkwitz [41] claims to have cultured the virus of measles both in tissue cultures and in symbiosis with bacteria, although he has not produced the typical disease in monkeys or human beings with these cultures. Serum from convalescent patients causes a passive immunity in those exposed to the disease and prevents an attack if injected dur-

ing the first few days of the incubation period. The weight of evidence seems to be in favor of a filterable virus as the etiological agent of measles.

The great epidemics of measles that swept through the army camps during the World War produced pulmonary lesions that resembled closely those observed in epidemic influenza. During ordinary times among the civilian population the pulmonary lesions following measles are much less severe. Nothing comparable to the acute edematous, hemorrhagic, pulmonary consolidation found in the early stage of influenza is seen in the ordinary case. Instead, the changes in measles are similar to those found in individuals who survive the early stage of influenza and begin to show interstitial bronchopneumonia. Although large areas of hemorrhagic consolidation are not found, a halo of hemorrhage frequently encircles the yellowish peribronchial nodules.

During the past five years about 30 autopsies have been performed by one of us upon children and young adults dying of measles, and practically every specimen shows a typical interstitial bronchopneumonia. Among these cases there were several instances of lobar pneumonia and others of marked confluent lobular pneumonia. Lung abscess and fulminating streptococcus pneumonia were rare. Pleurisy and empyema, which also occurred so often as complications of both influenza and measles in the army camps during the World War, are less frequently found following measles in the civilian population during peace times.

Microscopically the interstitial infiltration is marked, and collections of large mononuclear cells are found in the alveoli more frequently than in influenza. At times these cells form multinucleated giant cells. Dense masses of fibrin are also commonly seen in alveoli surrounding thickened bronchioles, and a polymorphonuclear exudate fills the bronchioles and some connecting alveoli in areas where bronchopneumonia is present.

The Etiology and Anatomical Lesions of Whooping Cough

A number of different bacteria were described as causing whooping cough prior to the isolation in 1906 by Bordet and Gengou [42] of the bacillus that is now generally accepted as the etiological agent. There are many apparently convincing reasons for regarding

the pertussis bacillus as the cause of this disease. In the first place the bacillus is associated with pertussis only, and is not found in other diseases. Bordet and Gengou first showed that this microorganism could be isolated from the bronchial secretions during the catarrhal stage of pertussis in a high percentage of cases, and that specific, complement-fixing antibodies were present in the blood of patients convalescent from pertussis. Chievitz and Meyer [43] soon confirmed these observations and since then others have amply corroborated them.

These clinical observations have never received the experimental confirmation that might be anticipated in a supposedly simple bacterial disease. The experiments of Klimenko [44] on young dogs are open to question because the possibility of distemper, which is frequent in these animals, was not ruled out and it is now known that *B. bronchisepticus* has biological characteristics much like those of the pertussis organism. Other workers have not succeeded in repeating Klimenko's results. The work of Mallory, Hornor and Henderson [45] called attention to the presence of bacilli between the cilia of the bronchial epithelium in pertussis. However, various types of bacteria can be demonstrated in the same position in other human diseases as well as in spontaneous infections in dogs, rabbits and guinea pigs.[46]

Some investigators have doubted the etiological rôle of the bacillus of Bordet and Gengou. Indeed a critical study of the relation of this bacillus to the disease reveals disturbing inconsistencies. The bacilli are present only in the early stages of the disease. The incubation period of pertussis is longer than that of the average bacterial disease. A vaccine prepared from the bacillus has been used for a number of years but is of doubtful value either as a prophylactic or as a curative agent. Because of this it was omitted from New and Non-Official Remedies in 1931.[47]

B. influenzae has often been isolated from cases of pertussis, although Winholt [48] claims that complement fixation tests with this organism are negative in such instances. In this connection it is interesting to recall that Jochmann and Krause,[49] who were the first to describe a specific organism as the cause of pertussis, described one that later proved to be a strain of *B. influenzae*.

In 1903, and again in 1913, Manicatide [50] advanced similar claims for a bacillus he described, and reported positive complement fixa-

tion with it in whooping cough convalescents and negative reactions in normal individuals.

Odaira [51] advanced the idea that pertussis is a symptom complex that may be caused by a variety of bacteria. This conclusion seems improbable however, because of the epidemiology of the disease and the development of a rather solid immunity after an attack.

Kraus [52] suggested that the primary cause of the disease was a filterable virus and claimed good results by treating patients with sterile filtrates of pertussis sputum. Convalescent serum has recently been used as a prophylactic with suggestive results by Bradford.[53]

Encephalitis is an infrequent complication of whooping cough, also of measles and vaccinia, although this is only remote evidence in favor of a virus since encephalitis has been known to follow other mild upper respiratory infections.

The recent discovery of intranuclear inclusions in about one-third of our autopsied cases of pertussis (McCordock [54]) is strong evidence in favor of the possible rôle of a filterable virus in this disease.

The pulmonary lesions of whooping cough are much the same as those of measles. Interstitial bronchopneumonia is usually quite marked and collections of large mononuclear cells are often found in the alveoli. When present, intranuclear inclusions serve to distinguish the interstitial pneumonia of whooping cough from that of measles and influenza. These inclusions are acidophilic, staining pink with Mallory's eosin-methylene blue, and with Giemsa's stain. A clear zone separates the inclusions from the nuclear membrane to which small particles of chromatin sometimes adhere. Inclusions are found in the cells lining the alveoli, in the bronchial epithelium and occasionally in the cells of the mucous glands or ducts of the bronchial walls. In a small percentage of cases inclusions are found in liver cells about which there are foci of necrosis with infiltration of polymorphonuclear leukocytes. They are also found in similar focal necroses in the adrenal gland. The cells containing the inclusions are larger than their neighbors and have a more basophilic cytoplasm. That these inclusions bear a specific relation to pertussis is evident, since they have been found in a third of all our cases of pertussis and only twice in 100 control autopsies of children dying of various other diseases. In both of these there was also a peribronchial infiltration with large mononuclear cells and lympho-

cytes. Genuine intranuclear inclusions have undoubtedly always been present in cases of pertussis, and are not spurious or of spasmodic occurrence, or restricted to a particular locality, since we have found them in sections from autopsies performed in St. Louis during the last 15 years and have observed them in autopsy material in other distant cities as well. Feyrter [55] of Hamburg has reported intranuclear inclusions in one case of pertussis, but this is the only other instance we have been able to find in the literature in which inclusions have been associated with whooping cough.

The pulmonary complications of pertussis are similar to those of measles. Severe, confluent lobular pneumonia, and the lobar type in older children, are the only common postpertussis lesions. In our experience severe bronchiectasis is more frequently encountered in this disease than in influenza or measles. This may in part be due to the more chronic nature of pertussis and to the violent paroxysms of coughing.

Discussion

The experimental work on influenza during and since the last pandemic amply illustrates the futility of attempting to explain the disease simply upon the basis of a bacterial infection. It would be unique for great epidemic outbursts of a disease like influenza to be caused by a bacterium that ordinarily is a harmless inhabitant of the nasopharynx of many healthy individuals at all times. Indeed the epidemic character of this malady resembles that of a virus disease such as measles or poliomyelitis, rather than that of a bacterial disease due to a microörganism that usually is an innocuous commensal but suddenly develops virulent characteristics.

While direct proof that influenza is caused by a filterable virus is still wanting, the character of the anatomical lesions observed in this disease furnishes indirect evidence in favor of such a view, since lesions similar to those of influenza are found in measles, a known virus disease, and can be produced experimentally in animals with vaccine virus. In accordance with this interpretation the influenza virus is probably pneumotropic and produces in the vast majority of individuals affected a severe, although self-limited, respiratory disease. Some patients succumb a few days after the onset of symptoms and the lungs of these individuals show a hemorrhagic and edematous lobular consolidation, a patchy destruction and desqua-

mation of alveolar and bronchial epithelium, and, at times, foci of necrosis in which all the pulmonary structures are killed *en masse.* This corresponds to Wolbach's first stage, or to the acute diffuse fulminating influenzal pneumonia of Winternitz. We have shown that lesions identical with these can be produced with vaccine virus. By itself this fact is of no significance, since many injurious substances can produce the same damage, as Winternitz has shown in experiments with war gases. The importance of vaccine virus in this connection, however, becomes at once apparent when it is recalled that if allowed to act in a more dilute form for a longer time the same virus incites the interstitial changes found in the later stages of influenza, and when it is used in combination with bacteria most of the complications of this disease can be reproduced in animals. This is far more than can be accomplished with either the war gases or with the influenza bacillus, no matter how much its virulence has been increased, in spite of the capacity of each of these agents to produce severe initial damage.

In the early stage of the disease the lungs often show in addition a bronchiolitis, and any bacteria present are found in the exudate within the bronchioles. However, at this time bacteria are scarce, as pointed out by Goodpasture, and consist principally of influenza bacilli and occasionally streptococci.

The majority of patients, in whom the disease is not of short duration, survive this initial period, subsequently developing more chronic virus lesions and one or more of a variety of pulmonary complications due to an assortment of secondary bacterial invaders. Interstitial bronchopneumonia is the most constant additional lesion in individuals who survive the first week. Three distinct elements can be recognized in the lesion that MacCallum has designated interstitial bronchopneumonia. Two of these, namely, the cellular infiltration of the interstitial tissue and the non-purulent peribronchial alveolar exudate composed of large mononuclear cells, dense compact masses of fibrin or red cells, we regard as a histological manifestation of the action of a virus upon the lung and include it in the description of pure virus pneumonia. Both of these changes can be produced experimentally with virus alone and they are also found in individuals dying of a virus disease, such as measles, in regions of the lung where bacteria cannot be demonstrated. The third element, the lobular or bronchopneumonia, is the

result of secondary bacterial infection that calls forth a polymorphonuclear cell exudate in the bronchioles and in groups of connecting alveoli.

The *Streptococcus hemolyticus*, *B. influenzae*, and other bacteria have been regarded as the cause of interstitial bronchopneumonia by different authors. Such a view seems unlikely, since the interstitial part of the lesion may occur in the absence of bacteria, and many examples of pulmonary infection with these bacteria exist without a suggestion of the interstitial infiltration. Again, many cases of measles and whooping cough present a well developed interstitial infiltration, and neither the influenza bacillus, the streptococcus, nor any other bacterium is constantly associated with this lesion.

The influenza bacillus has been found more frequently in each of these diseases associated with interstitial bronchopneumonia than any other organism. It will be recalled that Blake found it present in 80 per cent of the cases of measles during the influenza epidemic, and others have reported its presence in the same disease during interepidemic periods. Odaira,[51] Winholt,[48] Kristensen [56] and others have isolated the bacillus from cases of whooping cough. Little significance, however, can be attached to the presence of *B. influenzae* in these three diseases when its frequency in other conditions is considered. Working in Chicago over a 3 year period Bourn [57] showed that influenza bacilli could be demonstrated in about 50 per cent of individuals suffering from pneumonia of all types, in 40 per cent of patients with pulmonary tuberculosis, in 30 per cent with various other respiratory disorders, as well as in about 20 per cent of the healthy population. If *B. influenzae* were the cause of interstitial bronchopneumonia is it not remarkable that it should produce this lesion in three diseases only — influenza, measles and whooping cough — when it also is present in such a high percentage of other respiratory diseases? True interstitial bronchopneumonia has not been reproduced in animals by the injection of the influenza bacillus or other associated respiratory bacteria.

Some children dying of bronchial diphtheria show a cellular infiltration of the walls of affected bronchi, in addition to a lobular pneumonia. This reaction has occasionally been cited as an instance of interstitial bronchopneumonia caused by a bacterium. However, the cases of diphtheria we have studied can be distinguished easily

from those of measles or whooping cough. In diphtheria the infiltrating cells are practically all lymphocytes and plasma cells with polymorphonuclear leukocytes near the epithelial surface, while the large mononuclear wandering cells found so commonly in interstitial pneumonia are scarce. The groups of alveoli encircling infiltrated bronchi do not, as a rule, contain the dense compact masses of fibrin or the collections of large mononuclear cells, as do those in measles and whooping cough. Instead they are uniformly filled with the acute inflammatory exudate characteristic of lobular pneumonia. The diffusion of toxin into the bronchial wall from diphtheria bacilli growing upon the epithelial surface seems an adequate explanation of the cellular infiltration in this disease.

There is other indirect evidence substantiating the theory that the primary cause of interstitial bronchopneumonia is a virus. Recently we examined the lungs of a child who died of generalized vaccinia and found a typical interstitial bronchopneumonia, as well as abscesses which were due to a secondary staphylococcus infection. Through the courtesy of Dr. T. M. Rivers, sections of two cases of human psittacosis and one of an experimental infection in a monkey have been obtained. These also show the interstitial reaction and in the sections of one elements of the more acute hemorrhagic virus pneumonia can be made out. Several examples of interstitial bronchopneumonia in dogs, which showed respiratory complications during a spontaneous distemper infection, have been found in Roman's [58] material. These three diseases, as well as measles, are caused by viruses, and associated with each examples of interstitial bronchopneumonia can be found. The discovery of intranuclear inclusions in pertussis also points to the association of a virus with interstitial bronchopneumonia in whooping cough.

Specific bacteria have been in the past, or are at present, regarded by some workers as the etiological agents of influenza, measles and pertussis. If viruses are held to be the primary cause of these diseases the associated bacteria naturally will be regarded as secondary invaders. In the case of measles the majority of workers already accept such an interpretation. The studies initiated by the last pandemic have cast such grave doubt upon the causal relation of *B. influenzae* to epidemic influenza in man that few investigators still regard it as the cause of this disease. The pertussis bacillus, on the other hand, is almost universally regarded as the cause of whoop-

ing cough. The evidence in favor of this conclusion seems unassailable, and bacteriologists will point to the high incidence of the bacillus in this disease, its absence in other conditions and the presence of specific antibodies in the serum of convalescents. Indeed the bacillus may still have a very direct association with the disease, and future work alone will determine this relation. In the meantime, however, in connection with both influenza and pertussis, it is interesting to recall the history of three other diseases, namely, hog cholera, canine distemper and psittacosis, which for years were regarded as being caused by specific bacteria but now are known to be primarily due to viruses. If the virus nature of influenza and pertussis can eventually be proved by direct experiments, hog cholera and dog distemper will furnish perfect analogies of a similar revision in our conception of the etiology of a disease.

In 1886 Salmon and Smith [59] described *B. suipestifer* as the etiological agent of hog cholera. This organism was found almost constantly associated with the spontaneous disease and produced in experimental animals a condition which closely resembled the natural infection, even to the extent of showing similar pathological lesions. This work apparently so firmly established the etiological rôle of *B. suipestifer* that for 17 years it was universally regarded as the cause of hog cholera, just as the pertussis bacillus is today so universally thought to be the primary cause of whooping cough. At length, de Schweinitz and Dorset [60] in 1903 proved that the disease could be transmitted with bacteria-free filtrates of organs and blood from infected animals and that *B. suipestifer* was a common secondary invader.

The history of distemper repeats the same sequence of events. In this disease, as in pertussis, the almost constant presence of specific antibodies in the blood of convalescents seemed to establish beyond question the etiological rôle of the associated organism *B. bronchisepticus*. A vaccine was prepared and for a time enthusiastically used, but, like the pertussis vaccine, it eventually proved to be of doubtful value and was discarded. Finally, Roman [58] in 1925, and Dunkin and Laidlow [61] in 1926, proved that the disease was caused by a filterable virus.

In a study of swine influenza Shope [62] has shown that this disease is caused by a filterable virus and *H. influenzae suis* acting together. The injection of cultures of the bacillus alone is without

effect, and animals receiving only the filterable virus develop a mild "filtrate disease" which is not typical swine influenza. The pathological lesions of swine influenza[63] resemble those of human epidemic influenza. This work supplies additional experimental evidence in support of our conclusion that the pulmonary lesions of epidemic influenza, measles, and pertussis are in all probability due to the combined action of a filterable virus and bacteria.

Interstitial bronchopneumonia is regarded by us as the type reaction to the presence of a virus and a bacterium, but in no sense specific for a certain virus or a particular microörganism. Such a distinct lesion is usually type specific, and is caused by a particular stimulus or by several related stimuli, especially when the lesion is restricted to a group of related diseases. An acute inflammatory exudate is an example of a specific reaction that is called into play in the presence of pyogenic bacteria, and as such it is a useful index of infection even though sterile necrotic tissue or chemical irritants can induce the same reaction. A tubercle is the type reaction to the presence of foreign bodies in the tissue and although associated with a large variety of foreign bodies, tubercles, nevertheless, are helpful in the diagnosis of tuberculosis. However, tuberculosis can exist apart from tubercles or giant cells, as for instance in tuberculous meningitis or pneumonia, in which a large number of tubercle bacilli excite an exudative reaction which is essentially an acute inflammation. Similarly, we have shown that vaccine virus can produce two distinct types of reaction in the rabbit's lung. One has been designated as hemorrhagic virus pneumonia because it consists essentially of hemorrhage, edema and necrosis, and the other, interstitial virus pneumonia because of the marked accumulation of wandering cells in the interstitial tissue. The latter type of reaction is one of the histological components of interstitial bronchopneumonia in man and represents, we believe, the action of some virus upon the lung tissue. The lobular, or bronchopneumonic constituent of the lesion is the result of one or more of a variety of pyogenic bacteria entering the lung as secondary invaders after the virus has impaired the defense mechanisms that ordinarily prevent these microörganisms from infecting the lung. In this sense we regard interstitial bronchopneumonia as a type specific reaction due to the combined presence of a virus and bacteria.

Summary and Conclusions

Vaccine virus injected into the lungs of rabbits can incite two different types of reaction depending upon its concentration. A strong virus tends to produce a hemorrhagic, edematous consolidation and irregular areas of necrosis with hemorrhage that resemble infarcts. Polymorphonuclear leukocytes infiltrate the necrotic tissue if the animal survives. This acute reaction has been termed hemorrhagic virus pneumonia. On the other hand, a dilute virus calls forth a proliferative cellular lesion, which we have called interstitial virus pneumonia, in which the walls of the bronchi, the alveoli and the blood vessels become thickened due to an infiltration that consists principally of mononuclear cells.

The lesions of hemorrhagic virus pneumonia in rabbits are similar to the hemorrhagic and edematous lobular consolidation with foci of necrosis found in the lungs of individuals dying a few days after the onset of symptoms of epidemic influenza. The cellular infiltration of interstitial virus pneumonia in rabbits resembles the interstitial accumulation of cells seen in the interstitial bronchopneumonia that so frequently accompanies influenza, measles and whooping cough in man. It is impossible to reproduce the complete picture of interstitial bronchopneumonia with vaccine virus alone, for the lobular or bronchopneumonic component is always lacking, but it can be superimposed upon interstitial virus pneumonia by injecting bacteria into the lungs of a rabbit subsequent to the introduction of dilute vaccine virus. Not only can the complete picture of interstitial bronchopneumonia be reproduced by the combined use of vaccine virus and bacteria, but also practically all the pulmonary complications of influenza, measles and whooping cough, with the exception of lobar pneumonia.

Interstitial bronchopneumonia can be reproduced by vaccine virus and bacteria, and it also is found in known virus diseases such as measles, generalized vaccinia, psittacosis, and in some cases of spontaneous distemper in dogs that develop respiratory complications. Interstitial bronchopneumonia is therefore regarded as the type reaction for the combination of a virus and bacteria, although in no sense specific for a particular virus or bacterium.

Influenza and pertussis are the two human diseases associated with interstitial bronchopneumonia for which satisfactory proof of

the presence of a virus is lacking, although the type of the anatomical lesions associated with each is indirect evidence of the action of such an agent. In the case of pertussis the recent discovery of intranuclear inclusions in about one-third of our autopsied cases, and their absence in control material, support the idea that a virus is associated with interstitial bronchopneumonia and indicate that a filterable virus may also play a rôle in the cause of this disease.

REFERENCES

1. Cole, R., and MacCallum, W. G. Pneumonia at a Base Hospital. Part II. Pathology. *J. A. M. A.*, 1918, **70**, 1153–1156.
2. Bartels. Bemerkungen über eine im Frühjahre 1860 in der Poliklinik in Kiel beobachtete Masernepidemie, mit besonderer Berücksichtigung der dabei vorgekommenen Lungenaffectionen. *Virchows Arch. f. path. Anat.*, 1861, **21**, 65–84.
3. Delafield, F. The pathology of broncho-pneumonia. *Boston M. & S. J.*, 1884, **111**, 484–487.
4. MacCallum, W. G. The pathology of the pneumonia in the United States Army camps during the winter of 1917–18. *Monographs of the Rockefeller Institute for Medical Research*, No. 10, 1919.
5. Opie, E. L., Blake, F. G., Small, J. C., and Rivers, T. M. Epidemic Respiratory Disease. C. V. Mosby Co., St. Louis, 1921.
6. Honl, I. Zur Characteristik der Pneumonie nach Masern. *Wien. klin. Rundschau*, 1897, **11**, 833.
7. Muckenfuss, R. S., McCordock, H. A., and Harter, J. S. A study of vaccine virus pneumonia in rabbits. *Am. J. Path.*, 1932, **8**, 63–71.
8. Park, W. H. Bacteriology of recent pandemic of influenza and complicating infections. *J. A. M. A.*, 1919, **73**, 318–321.
9. Wolbach, S. B. Comments on the pathology and bacteriology of fatal influenza cases, as observed at Camp Devens, Mass. *Bull. Johns Hopkins Hosp.*, 1919, **30**, 104–109.
10. Pritchett, I. W., and Stillman, E. G. Occurrence of *Bacillus influenzae* in throats and saliva. *J. Exper. Med.*, 1919, **29**, 259–266.
11. Spooner, L. H., Scott, J. M., and Heath, E. H. A bacteriologic study of the influenza epidemic at Camp Devens, Mass. *J. A. M. A.*, 1919, **72**, 155–159.
12. McIntosh, J. The incidence of *Bacillus influenzae* (Pfeiffer) in the present influenza epidemic. *Lancet*, 1918, **2**, 695–697.
13. Messerschmidt, T., Hundeshagen, K., and Scheer, K. Untersuchungen über die Influenzaepidemie, 1918. *Ztschr. f. Hyg.*, 1918, **88**, 552–571.
14. Camp Lewis Pneumonia Unit. The relation of bronchopneumonia to influenza. *J. A. M. A.*, 1919, **72**, 268–269.

15. Kinsella, R. A. Bacteriology of epidemic influenza and pneumonia. *J. A. M. A.*, 1919, **72**, 717–720.

16. Hirsch, E. F., and McKinney, M. Epidemic of bronchopneumonia at Camp Grant, Ill. *J. A. M. A.*, 1918, **71**, 1735–1736.

17. Williams, A. W., Nevin, M., and Gurley, C. R. Studies on acute respiratory infections. I. Methods of demonstrating microörganisms, including "filterable viruses" from upper respiratory tract in "health" in "common cold" and in "influenza" with the object of discovering "common strains." *J. Immunol.*, 1921, **6**, 5–24.

18. Wollstein, M. The influenza bacillus in inflammations of the respiratory tract in infants. *J. Exper. Med.*, 1906, **8**, 681–691.

19. Davis, D. J. The bacteriology of whooping-cough. *J. Infect. Dis.*, 1906, **3**, 1–36.

20. Liebscher, C. Ueber Influenzabacillen-Befunde bei Masern- und Scharlacherkrankungen. *Prag. med. Wchnschr.*, 1903, **28**, 85–99.

21. Boggs, T. R. The influenza bacillus in bronchiectasis. *Am. J. M. Sc.*, 1905, **130**, 902–911.

22. Lord, F. T. Infections of the respiratory tract with influenza bacilli and other organisms, their clinical and pathological similarity, and confusion with tuberculosis. *Boston M. & S. J.*, 1905, **152**, 537–540.

23. Medical Research Committee of Great Britain. Influenza. *Medical Supplement*, Oct. 1, 1918. Abstr. *J. A. M. A.*, 1918, **71**, 1573–1579.

24. MacCallum, W. G. Pathology of the pneumonia following influenza. *J. A. M. A.*, 1919, **72**, 720–723.

25. Blake, F. G., and Cecil, R. L. Studies on experimental pneumonia. Production in monkeys of an acute respiratory disease resembling influenza by inoculation with *Bacillus influenzae*. *J. Exper. Med.*, 1920, **32**, 691.

Cecil, R. L., and Blake, F. G. Studies on experimental pneumonia. Pathology of experimental influenza and of *Bacillus influenzae* pneumonia in monkeys. *J. Exper. Med.*, 1920, **32**, 719.

26. Parker, J. T. The poisons of the influenza bacillus. *J. Immunol.*, 1919, **4**, 331–357.

27. Fildes, P., and McIntosh, J. The aetiology of influenza. *British J. Exper. Path.*, 1920, **1**, 119–126; 159–174.

28. Huntoon, F. M., and Hannum, S. The rôle of *Bacillus influenzae* in clinical influenza. *J. Immunol.*, 1919, **4**, 167–187.

29. Winternitz, M. C., Wason, I. M., and McNamara, F. P. The Pathology of Influenza. Yale University Press, New Haven, 1920.

30. Gibson, H. G., Bowman, F. B., and Connor, J. I. The etiology of influenza: a filterable virus as the cause. With some notes on the culture of the virus by the method of Noguchi. *Medical Research Committee, Great Britain, Report Series No. 36*, London, 1919, 19.

A filterable virus as the cause of the early stage of the present epidemic of influenza. *Brit. M. J.*, 1918, **2**, 645–646.

31. Nicolle, C., and Lebailly, C. Recherches expérimentales sur la grippe. *Ann. de l'Inst. Pasteur*, 1919, **33**, 395–402.

32. Yamanouchi, T., Sakakami, K., and Iwashima, S. The infecting agent of influenza; an experimental research. *Lancet*, 1919, **1**, 971.

33. Lister, F. S., and Taylor, E. Experimental investigation of epidemic influenza at Durban. *Publ. S. African Inst. M. Research*, 1919, No. 12, 9–13.

34. Goodpasture, E. W., and Burnett, F. L. The pathology of pneumonia accompanying influenza. *U. S. Nav. M. Bull.*, 1919, **13**, 177–197.

35. Tunnicliff, R., and Hoyne, A. L. Further studies on a diplococcus from measles. Prevention of measles by immune goat serum. *J. Infect. Dis.*, 1926, **38**, 48–53.

36. Long, P. H., and Cornwell, A. M. Observations on the etiology of measles. *J. Infect. Dis.*, 1927, **40**, 408–411.

37. Bradford, W. L. Skin reactions to filtrates and killed cultures of green-producing cocci in relation to measles. *J. Infect. Dis.*, 1929, **44**, 378–382.

38. Smith, J. Green-producing cocci in measles. *J. Hyg.*, 1928–1929, **28**, 363–375.

39. Hektoen, L. Experimental measles. *J. Infect. Dis.*, 1905, **2**, 238–255.

40. Blake, F. G., and Trask, J. D., Jr. Studies on measles. I. Susceptibility of monkeys to the virus of measles. *J. Exper. Med.*, 1921, **33**, 385–412; 413–422; 621–626.

41. Degkwitz, R. The etiology of measles. *J. Infect. Dis.*, 1927, **41**, 304–316.

42. Bordet, J., and Gengou, O. Le microbe de la coqueluche. *Ann. de l'Inst. Pasteur*, 1906, **20**, 731–741.

43. Chievitz, I., and Meyer, A. H. Recherches sur la coqueluche. *Ann. de l'Inst. Pasteur*, 1916, **30**, 503–525.

44. Klimenko, W. N. Die Aetiologie des Keuchhusten. *Centralbl. f. Bakteriol.*, Pt. 1, Orig. 1908, **48**, 64–76.

45. Mallory, F. B., Hornor, A. A., and Henderson, F. F. The relation of the Bordet-Gengou bacillus to the lesions of pertussis. *J. Med. Res.*, 1913, **27**, 391–398.

46. Rhea, L. J. The comparative pathology of the tracheal and bronchial lesions produced in man by *B. pertussis* (whooping cough) and those produced in dogs by *B. bronchisepticus* (canine distemper). *J. Med. Res.*, 1915, **32**, 471–474.

47. Council on Pharmacy and Chemistry. Pertussis vaccines omitted from New and Non-Official Remedies. *J. A. M. A.*, 1931, **96**, 613.

48. Winholt, W. Complement-fixation in whooping cough. *J. Infect. Dis.*, 1915, **16**, 389–398.

49. Jochmann, G., and Krause, P. Zur Aetiologie des Keuchhustens. *Ztschr. f. Hyg. u. Infectionskr.*, 1901, **36**, 193–222.

50. Manicatide, M. Ueber Aetiologie und Serotherapie des Keuchhustens. *Ztschr. f. Hyg. u. Infectionskr.*, 1903, **45**, 469–506.

Der Komplementbindungsvorgang bei Keuchhusten. *Zstchr. f. Kinderh.*, 1913, **7**, Orig., 226–232.

51. Odaira. Beiträge zur Kenntnis der hämoglobinophilen Bacillen, mit besonderer Berücksichtigung des Bordetschen Bacillus. *Centralbl. f. Bakteriol.*, Pt. 1, Orig. 1912, **61**, 289–308.

52. Kraus, R. Eine neue Behandlungsmethode des Keuchhustens. *Deutsch. med. Wchnschr.*, 1916, **42**, 281–283.

53. Bradford, W. L. Private communication.

54. McCordock, H. A. Intranuclear inclusions in pertussis. *Proc. Soc. Exper. Biol. & Med.*, 1932, **29**, 1288–1291.

55. Feyrter, F. Über die pathologische Anatomie der Lungenveränderungen beim Keuchhusten. *Frankfurt Ztschr. f. Path.*, 1927, **35**, 213–255.

56. Kristensen, M. Investigations into the occurrence and classification of the haemoglobinophilic bacteria. Inaug. Diss., Copenhagen, 1922.

57. Bourn, J. M. Incidence of Pfeiffer's bacillus in throats during epidemic and interepidemic periods in Chicago. *J. Prev. Med.*, 1928, **2**, 441–460.

58. Roman, B. Lesions of the central nervous system in canine distemper. *Bull. Buffalo General Hosp.*, 1925, **3**, 40.

59. Salmon, D. E., and Smith, T. *Am. Monthly Microscop. J.*, 1886. Investigations in swine plague. *U. S. Dept. Agric., Bureau Animal Ind.*, 1885, 90.

60. de Schweinitz, E. A., and Dorset, M. A form of hog cholera not caused by the hog-cholera bacillus. *U. S. Dept. Agric., Bureau Animal Ind., Washington*, 1903. Circular 41.

61. Dunkin, G. W., and Laidlow, P. P. Studies in dog-distemper. *J. Comp. Path. & Therap.*, 1926, **39**, 201; 213; 222.

62. Shope, R. E. Swine Influenza. III. Filtration experiments and etiology. *J. Exper. Med.*, 1931, **54**, 373–385.

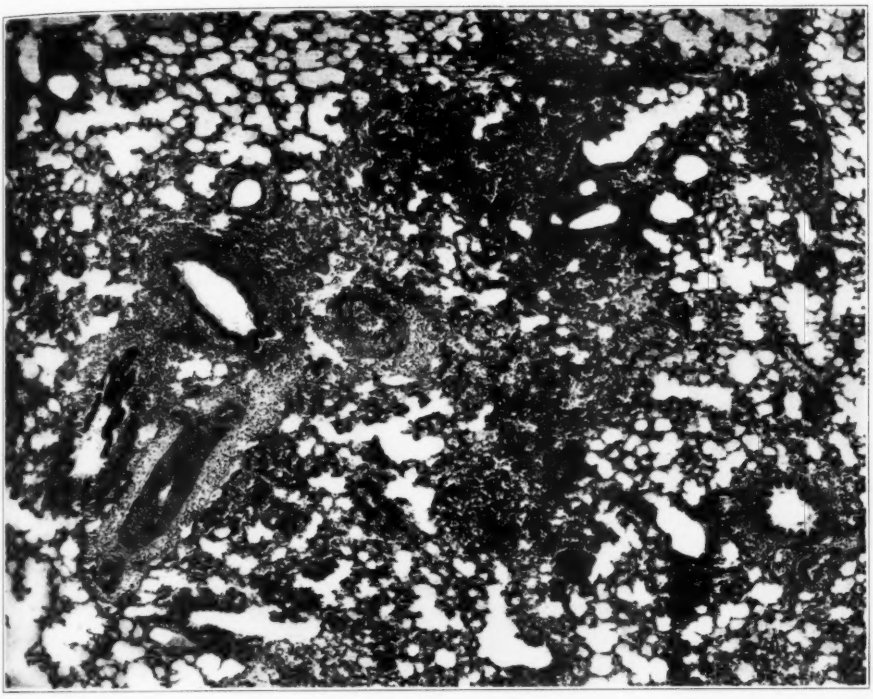
63. Shope, R. E. Swine Influenza. I. Experimental transmission and pathology. *J. Exper. Med.*, 1931, **54**, 349–359.

DESCRIPTION OF PLATES

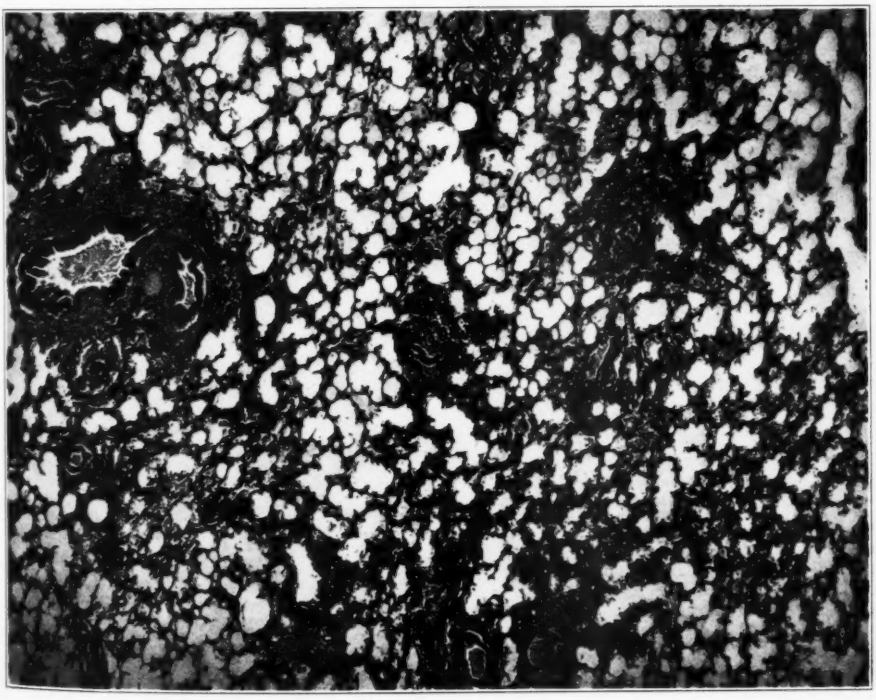
PLATE 32

FIG. 1. Interstitial virus pneumonia in a rabbit 5 days after inoculation of dilute vaccine virus. A zone of infiltration encircles each bronchus and many of the alveolar walls are thickened. No bacteria could be demonstrated in this lung. × 30.

FIG. 2. Interstitial bronchopneumonia following measles in a child. Note the peribronchial infiltration and the thickening of many of the alveolar walls. Most of the bronchi contain an exudate composed of polymorphonuclear leukocytes, fibrin and bacteria, which is not present in the animal lung illustrated above. × 30.



1



2

McCordock and Muckenfuss Virus Pneumonia in Animals

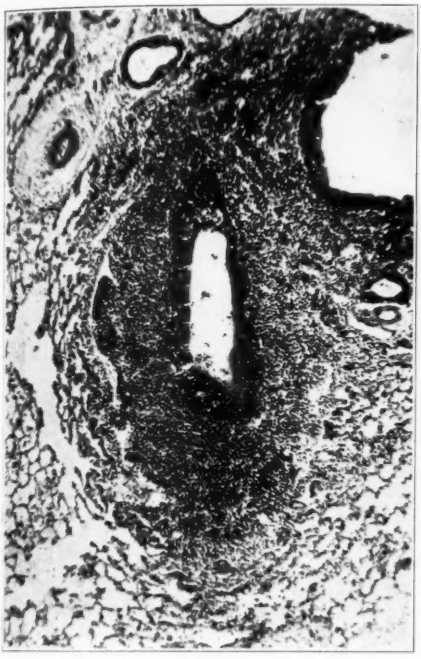
PLATE 33

FIG. 3. Bronchiole of animal lung 7 days after injection of virus. The peribronchial tissue is densely infiltrated with mononuclear cells. The lumen is clear. × 80.

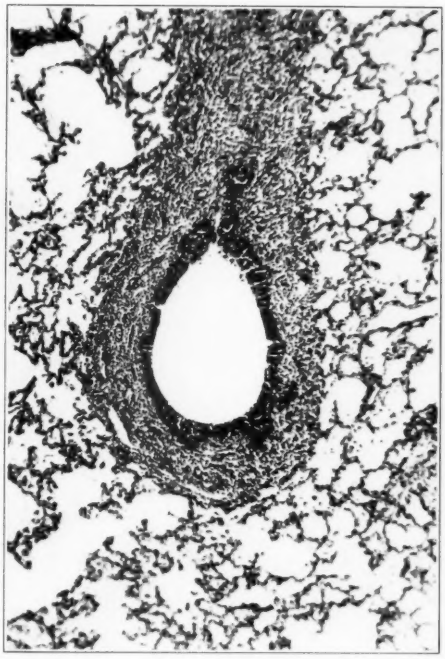
FIG. 4. Similar peribronchiolar infiltration in the lung of a child who died of pertussis. No bacteria were demonstrated in sections from this region of the lung. × 80.

FIG. 5. Peribronchial infiltration and polymorphonuclear exudate in the lumen of the bronchiole. Lung of animal after the injection of virus and bacteria. × 80.

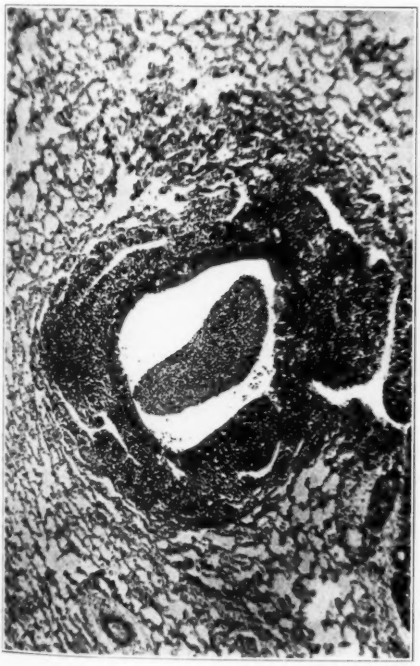
FIG. 6. Bronchiole from the lung of a child who died of pertussis showing lesions similar to those illustrated in Fig. 7. Bacterial stains of adjacent sections contain Gram-negative bacilli and Gram-positive cocci. Cultures of this lung revealed *B. influenzae* and a hemolytic staphylococcus. × 80.



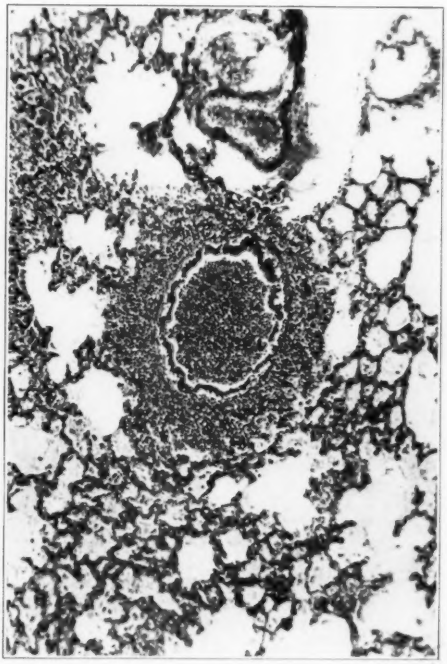
3



4



5



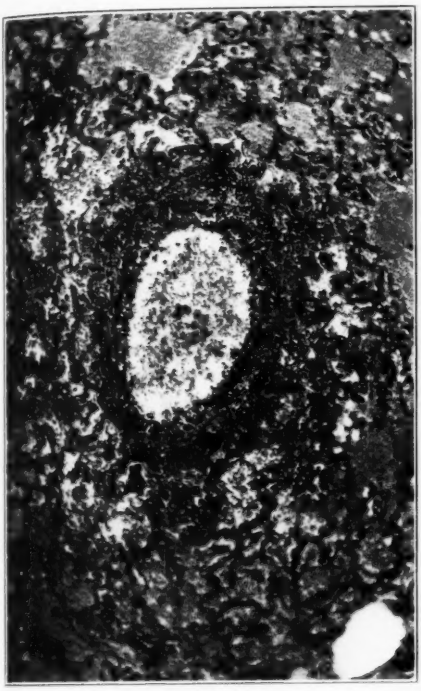
6

PLATE 34

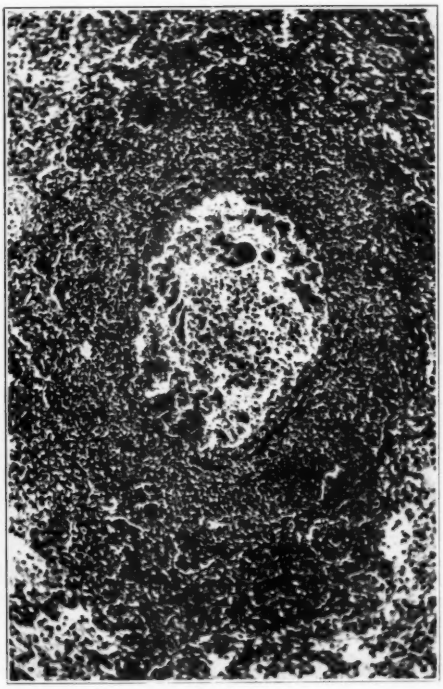
FIG. 7. Necrosis of the wall of a bronchiole in an animal after the injection of vaccine virus and bacteria. × 100.

FIG. 8. Necrosis of the wall of a small bronchus from a case of epidemic influenza. × 100.

FIG. 9. Experimental interstitial bronchopneumonia in an animal after the injection of virus and bacteria. There is a dense collection of mononuclear cells about the two bronchi shown in the lower part of the print. The lumen of each bronchus contains an exudate composed of polymorphonuclear cells, fibrin and bacteria. The lungs of this animal showed a lobular pneumonia that in places was confluent, as in the portion illustrated, in which all the alveoli are filled with a typical polymorphonuclear exudate. × 25.



7



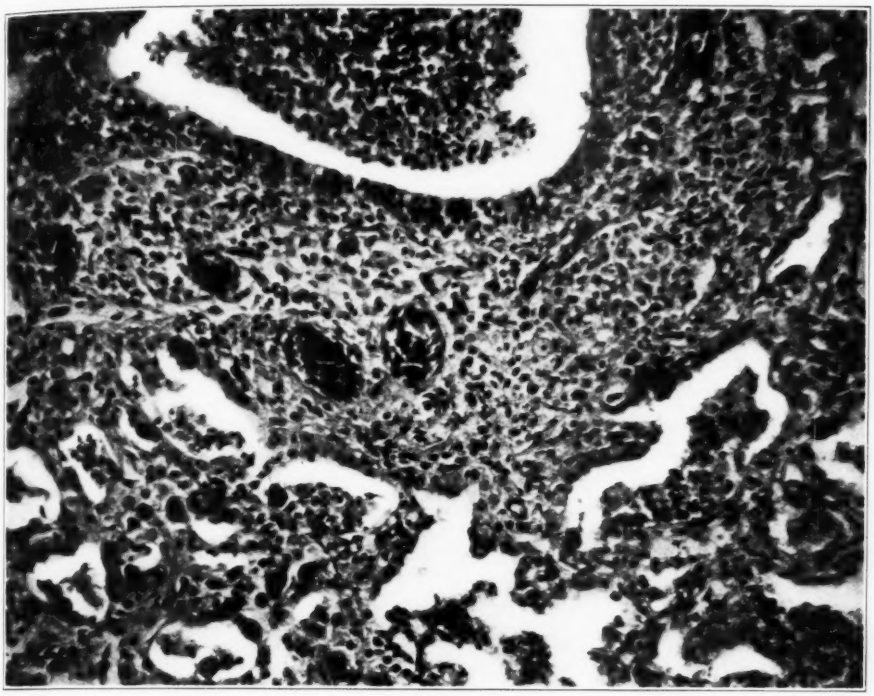
8

9

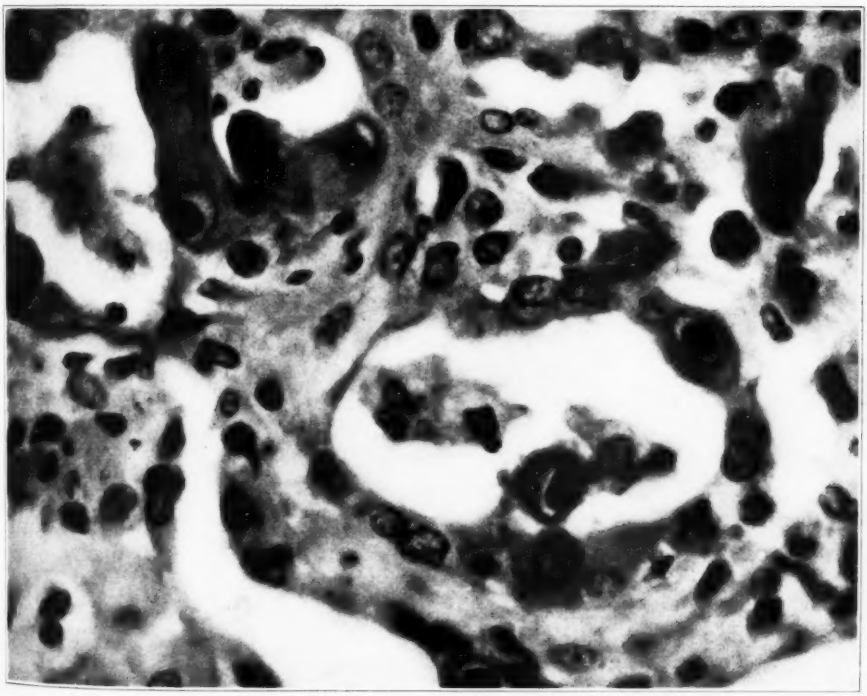
PLATE 35

FIG. 10. Interstitial bronchopneumonia in the lung of a child who died of pertussis about 3 weeks after the onset of symptoms. The peribronchial and alveolar wall thickening and the cellular infiltration are conspicuous. A leukocytic exudate is present in the lumen of the bronchiole. In the lower right-hand portion of the field intranuclear inclusions can be seen in the cells lining the alveoli. A portion of this region is shown in a higher magnification in Fig. 11. × 300.

FIG. 11. The intranuclear inclusions of pertussis. The alveolus in the lower half of the field and to the left contains a detached cell with an inclusion, but elsewhere the inclusions occur in cells lining the alveoli. × 850.



10



11

McCordock and Muckenfuss Virus Pneumonia in Animals

MICROTECHNICAL DEMONSTRATION OF INSOLUBLE LIME SALTS IN TISSUES *

G. GÖMÖRI, M.D.

(From the Third Surgical Clinic of the Royal Hungarian Pázmány Péter University of Budapest, Budapest, Hungary)

With microtechnical methods hitherto described insoluble lime salts can be demonstrated only if the tissue to be examined is soft enough for the microtome knife, *i.e.*, if it contains only small quantities of finely dispersed calcareous material. Under such conditions the Kóssa [1] silver impregnation or the Roehl lead sulphide reaction yields very instructive pictures. If, however, the tissue can be made sectionable only by decalcification, lime salts no longer can be demonstrated as they have been removed by the decalcifying agent. There are some methods for staining tissues which have contained calcium salts, *e.g.*, the simple hematoxylin stain, which imparts a characteristic dull dark blue color to decalcified tissues, or the Antonow [2] safranin-light green stain, and several others. They allow a fair orientation as to distribution of lime salts in the tissues, but they are, however, entirely unsuitable for more exact morphological research. It has been recommended, also, to make sections of hard tissue without decalcification, as even chips obtained in this way can be used for the Kóssa or Roehl method. By this method, however, as Antonow strikingly remarks, lime salts are liable to be demonstrated more readily by notches of the microtome knife than by staining.

With the method to be described insoluble lime salts of tissues can be demonstrated even in the hardest tissues in a very clear and selective manner, and in this way a relatively unknown field of normal and pathological histology is opened for scientific research.

I should like to describe the method more fully than is customary with new microtechnical methods, as it belongs to that small group of microtechnics which have a firm chemical basis.

It has the following basic principle: the lime salts must be stained or impregnated in blocks of fixed tissue before decalcification and

* Read before the First Conference of the Association of Hungarian Pathologists, Budapest, June 6th and 7th, 1932.

Received for publication August 29, 1932.

sectioning. Once the lime salts are stained, the tissues can be decalcified and embedded by routine methods. The stain remains at the site of the lime salts and if the reaction used is selective and to a certain extent quantitative, equivalent pictures must result, in the sense of Nissl.

Silver salts seemed to be quite suitable for the purpose mentioned. The lime salt content of bones and other calcareous deposits consists for the greater part of calcium phosphate and, to a lesser degree, of calcium carbonate. As silver phosphate is much less soluble than calcium phosphate, and silver carbonate also considerably less soluble than calcium carbonate, it is to be expected that if tissue blocks containing lime salts are put into a solution of silver nitrate a reaction will take place between the calcium and silver salts, with the result that calcium will be supplanted by silver, at least in the phosphate part, in an inverse proportion to the solubility of calcium and silver phosphate, respectively. This supposition has been verified by the experiments of P. Spanyár, chemical engineer. He poured an excess of a 0.1 normal silver nitrate solution upon accurately weighed quantities of calcium phosphoricum dibasicum "Kahlbaum" (a German brand of chemically pure dibasic calcium phosphate). The white powder at once became lemon yellow. After two days the silver nitrate solution was titrated and it could be stated that about 96 per cent of Ca had become supplanted by Ag.

The silver phosphate thus obtained was then reduced to metallic silver. Then the lime salts that did not partake in the reaction were removed by decalcification. The blocks thus treated were embedded and cut. In the sections black metallic silver was seen at the site of the lime salts.

This was the chemical basis on which I developed a practical procedure. Several hundred blocks of tissue containing insoluble lime salts (bones, sclerotic vessels, calcified tumors, tuberculomata) have been utilized. I shall first describe the method in detail and then describe the results.

Technical Method

1. Cutting of Fresh Tissues for Fixation: Blocks of tissue should not be thicker than 1 or 2 mm. The thinner the block, the better the result. Thicker blocks will remain unstained in their central parts.

2. *Fixation:* Formaldehyde seemed *a priori* to be unsuitable, as it itself reduces silver salts and in this way the blocks are prone to contain precipitated silver granules. On the other hand it always contains traces of formic acid, by which lime salts are easily dissolved. In general, no acid fixing fluids should be used because of the effect just mentioned. Salts of heavy metals are unsuitable because their phosphates likewise are insoluble. All bichromates and chlorides must be avoided too, in view of the later silver impregnation. There remain only three fixing methods: alcohol, acetone, and boiling. All three methods yield satisfactory results. I prefer, however, alcohol. Blocks should be kept for 2 to 4 days in 80 to 96 per cent ethyl or methyl alcohol. All dilution should be made with distilled water. Because of its strongly shrinking and hardening properties acetone is better not used. For boiling, a 2 per cent potassium nitrate or a 5 per cent alum solution in 20 per cent alcohol can be used. Aqueous solutions of these salts are not suitable for this method, as in distilled water calcium phosphate is markedly soluble (11 mg. per 100 cc.), whereas its solubility in 20 per cent alcohol is negligible (less than 1 mg. per 100 cc. (Spanyár)). If boiling is preferred, the blocks are dropped into the boiling solution and kept in it for 5 minutes.

After fixation blocks are washed in distilled water for 3 to 4 hours.

3. *Impregnation:* A 1.5 per cent solution of silver nitrate is used. Blocks are kept in it for 6 to 10 days at room temperature. Stronger solutions act more quickly, but they are prone to darken the blocks. Higher temperatures have the same effect. A gauze pad is put under the blocks in order to facilitate penetration by the solution. The silver solution is to be changed once or twice. The harder the block on account of its lime salt content, the longer the time required for impregnation. In the silver solution the parts containing lime salts become lemon yellow within a few minutes (silver phosphate). This color changes in several days into a pale brownish yellow. The tissues ought not to become darker, particularly not in their calcium-free parts. If, nevertheless, they should get a darker brown color, which almost never happens within 14 days, impregnation must be stopped.

4. *Washing of Tissues:* Now the blocks are thoroughly washed in large quantities of distilled water, changed 4 to 5 times daily for 3 to 4 days, until the last portion of decanted washing water does not show the slightest turbidity when mixed with hydrochloric acid. This

is very essential as blocks insufficiently washed are liable to become filled with a black-brown, granular precipitate.

5. *Reduction:* The following substances have been tried: photographic developers (such as metol, hydroquinone, pyrogallic acid, amidol), rongalite, neoarsphenamine, dextrose, formaldehyde, sodium nitrate, sodium sulphite and sodium hypophosphite. Of these, the photographic developers, rongalite and neoarsphenamine are too violent in their action. They reduce not only the inorganic silver salts but also the albuminates. The blocks become quite dark and the sections thus obtained are very dirty, loaded with an amorphous black precipitate. Dextrose, even in an alkaline medium, has a too weak reducing power. Formaldehyde has the drawbacks both of the photographic developers and of dextrose, as it is too weak and not selective in its action. Sodium nitrate does not reduce at all. Sodium sulphite yields very selective, sharp pictures, but the blocks are liable to contain a heavy precipitate. This is explained by the fact that many insoluble silver salts are dissolved in an excess of sodium sulphite, a complex sodium silver sulphite anion being formed. The latter is rather unstable and very soon metallic silver is precipitated from the solution. A most ideal reducer seems to be sodium hypophosphite. It reduces with an almost absolute selectivity only inorganic silver phosphate and carbonate, whereas it does not act upon albuminates. This is all the more interesting as it is difficult to obtain reduced silver phosphate by hypophosphites in the test tube when using pure chemicals. If a hypophosphite solution is poured over silver phosphate precipitated upon chemically pure calcium phosphate no changes will be observed for days; darkening begins much later and progresses but slowly. It has been noticed by Kóssa that silver phosphate formed from the calcium phosphate of tissues shows a different behavior from that formed from chemically pure calcium phosphate, the former being incomparably more light-sensitive than the latter. He assumes the possibility that the reaction described by him is due not to phosphates, but to an albuminous contamination. It is not easy to solve this problem. In any case the test tube experiment described above cannot be materially hastened by admixture of serum (author's own experiment). It seems to be most probable that a difference of dispersion plays an important rôle. Light sensitiveness of silver bromide emulsions likewise depends to a high degree on the amount of dispersion.

Sodium hypophosphite is used in a 5 per cent aqueous solution. The solution should be prepared fresh and made slightly alkaline by adding 4 to 5 drops of 0.1 normal sodium hydroxide solution to each 100 cc. of the fluid to counteract its acidity, as silver phosphate is markedly dissolved even by weak acids. Selective blackening of the calcareous parts can be noticed after about 30 minutes and reduction is completed within 4 to 8 days, depending on the thickness of the blocks. Sometimes the hypophosphite solution becomes brown. In this case it should be replaced by a fresh solution. It may not show, however, even the slightest turbidity, as this indicates insufficient washing of the blocks after impregnation. The result will be a blurring precipitation. The blocks themselves, except the calcareous parts which become black, do not change their color during reduction, or at best they become pale brownish.

6. After reduction the blocks are washed once more in running water for 3 to 4 hours.

7. Fixation for 2 days in a 3 to 5 per cent solution of sodium thiosulphate. The solution should be changed once or twice. All procedures mentioned hitherto beginning with impregnation should be performed in the dark. After fixation, however, the blocks have become insensitive to light and they can be treated further in daylight.

8. Washing in running water for at least 24 hours.

9. Decalcification: For this purpose acids which attack metallic silver (nitric acid, and to a lesser degree hydrochloric acid) must be discarded. The best is a 6 to 8 per cent solution of sulphosalicylic acid, which, upon my suggestion, has been used as a decalcifying agent for a number of years at the First Institute for Pathological Anatomy of the University of Budapest, giving great satisfaction. It does not attack metallic silver, nor is nuclear staining materially impaired by it. Decalcification is accomplished within 1 to 3 days, according to the thickness or the hardness of the blocks. It is advisable to change the acid solution once or twice. After decalcification blocks should be washed for the last time thoroughly.

This is the procedure proper. The blocks are now embedded by one of the routine methods. They are easily cut and can be stained by most staining methods.

Discussion

In the finished sections lime salts appear with a striking clearness a deep black on an entirely unstained background. Sometimes the most bizarre formations are observed such as (*a*) parallel arranged bulky disks lying at right angles to the connective tissue strands so as to form rows, connected by very fine tendril or branch-like filaments, (*b*) often spider or milliped-like figures, and (*c*) sometimes nothing but amorphous granules (see illustrations). Here I should like to mention that according to Schmorl [3] the Kóssa silver stain is unsuitable for study of the finer structures, as silver phosphate is a crystalline precipitate. I find that this objection is not justified. The fine tendril-like formations speak against composition of coarser crystals. Moreover, we have the Golgi impregnation which is based on the formation of a silver bichromate precipitate. If one mixes a drop of potassium bichromate and a drop of silver nitrate on a glass slide, one can observe under the microscope how coarse the crystals thus formed are. And yet nobody would condemn as being unsuitable for finer microscopic work the Golgi impregnation method which yields the most admirable results in neurohistology.

I am obliged to mention a shortcoming of my method and this is the relatively slight penetrating power of the impregnation. It often happens even after 14 days impregnation that only a more or less broad marginal zone of the thicker calcareous masses becomes impregnated, whereas the center remains unstained. This phenomenon has probably its underlying cause in the extremely slight permeability of the silver phosphate membrane which is formed upon the surface of the calcareous parts by the silver nitrate solution. A somewhat similar phenomenon is observed with bones fixed in alcohol when stained with hematoxylin: the characteristic dull dark blue color is often shown only by a thin marginal zone. Schmorl is disposed to explain this fact by chemical factors. He admits, however, that this explanation is not entirely satisfactory. The following facts furnish evidence that it is due entirely to the unsatisfactory permeation by the silver nitrate solution: (*a*) the longer the impregnation, the broader the stained zone; (*b*) that there is no question of unsatisfactory permeation by the reducing agent, which could be very well possible on account of the probably extremely slight permeability of the metallic silver membrane formed, has been demon-

strated by the following experiment. A great number of blocks have been impregnated for 3 to 14 days. One-half of these have been reduced by the method described. The other half have been directly decalcified with hydrochloric acid after thorough washing. The embedded blocks have been cut and the sections reduced in a hydroquinone solution. No other difference could be observed between sections prepared in these two different ways except that those decalcified with hydrochloric acid were exceedingly dirty and crowded with an amorphous black precipitate. The breadth of the stained zones, however, was exactly the same.

I think, however, that the shortcoming described cannot be considered as a grave defect. The pathologist is generally much more interested in the peripheral zone of the calcified foci, where calcification or halisteresis is active, than in the central parts where he can see for the most part only dead, inert material. On the other hand, at the boundary of the unstained, deeper layers the silver deposit is so densely black and so opaque that no structure whatever can be observed there, even in the case of a perfect permeation.

Attention is called to the fact that this method is suitable for demonstration of lime salts also in gross specimens, osteoplastic tumors and so on. Slices cut or sawed out from the material are fixed in alcohol, then kept for several hours in a silver nitrate solution until an intense lemon yellow color appears throughout the calcareous parts. After this the slices are thoroughly rinsed in distilled water and reduced in the hypophosphite solution for 24 hours. After fixing them in a thiosulphate solution and washing once more, they can be preserved in the fluids generally used for this purpose. The calcareous parts with their blackness on a background of natural colors give a very clear, striking picture.

Finally I wish to remark that preparations, microscopic as well as macroscopic, made according to my method are very durable. They withstand even direct sunshine for a long time.

Summary of Method

1. Cut or saw thin blocks of fresh tissue 1 to 2 mm. thick.

2. Fix in 80 to 96 per cent alcohol, or boil in one of the solutions mentioned. After this wash blocks in distilled water for 3 to 4 hours.

3. Impregnate in 1.5 per cent silver nitrate solution for 6 to 10 days. Change silver solution once or twice.

4. Wash for 3 to 4 days in distilled water changed daily 4 to 5 times, until the last washing water decanted does not show the slightest turbidity when mixed with hydrochloric acid.

5. Reduce in a 5 per cent solution of sodium hypophosphite. Before use add 4 to 5 drops of a 0.1 normal sodium hydroxide solution to each 100 cc. of reducer. Keep blocks in reducer for 4 to 8 days.

6. Wash in running water for 3 to 4 hours.

7. Fix in a 3 to 5 per cent solution of sodium thiosulphate for 2 days.

8. Wash in running water for at least 24 hours.

9. Decalcify in a 6 to 8 per cent solution of sulphosalicylic acid.

10. Wash, embed, and so on.

Steps 3 to 7 inclusive are to be performed in the dark.

REFERENCES

1. Kóssa, J. Über die im Organismus künstlich erzeugbaren Verkalkungen. *Beitr. z. path. Anat. u. z. allg. Pathol.*, 1901, **29**, 163–202.

2. Antonow, A. Eine einfache Methode für histologischen Kalknachweis. *Ztschr. f. wissensch. Mikr.*, 1926–27, **43**, 243–245.

3. Schmorl, C. G. Die pathologisch-histologischen Untersuchungsmethoden. F. C. W. Vogel, Leipzig, 1928, 194–197.

DESCRIPTION OF PLATES

PLATE 36

FIG. 1. Calcifying fibrochondrosarcoma of femoral condyles. × 50.

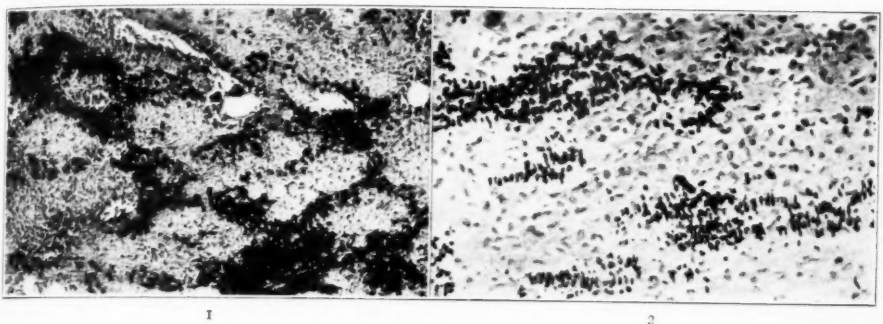
FIG. 2. Another part of the same tumor. × 100.

FIG. 3. Another part of the same tumor. × 300.

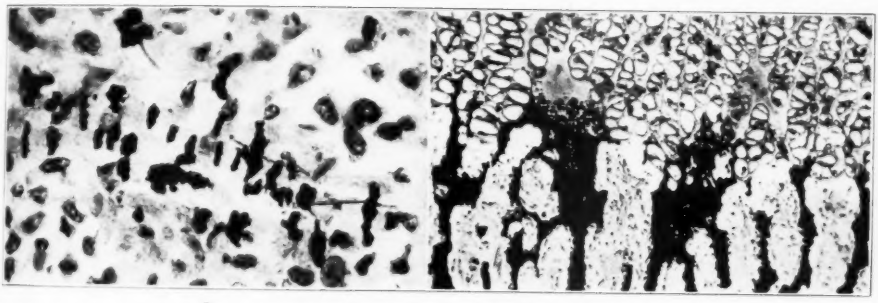
FIG. 4. Zone of ossification of lower femoral epiphysis in a newborn. × 50.

FIG. 5. Sclerotic patch of femoral artery. × 120.

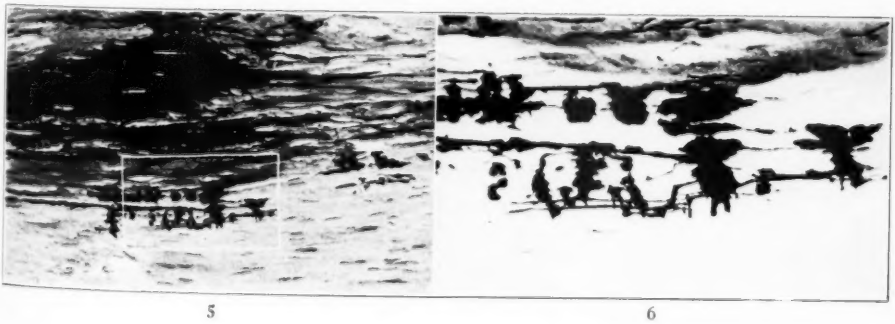
FIG. 6. The part of former figure outlined. × 350.



1 2



3 4



5 6

PIGMENT DEPOSITS IN INTESTINAL MUSCLE COATS AND THEIR RELATION TO DIET FACTORS *

EDMUND NACHTNEBEL, M.D.

HUNGARIAN SCHOLARSHIP COUNCIL FELLOW IN PATHOLOGY

(From the Department of Pathology, The University of Rochester School of Medicine and Dentistry, Rochester, N. Y.)

Some years ago Whipple and Hooper [1, 2] observed in a series of bile fistula dogs a peculiar brown or buff coloration of the muscle coats of the intestine. It was described as a "maple sugar color" and was most intense in the jejunum, fading a little in the ileum and still more in the colon. A similar pigmentation involved the stomach wall and pancreas in some cases. No increase in connective tissue was ever observed related to this pigment deposit and no positive stains for iron found. At that time it was suspected that this pigmentation was related to the abnormalities observed by Wisner and Whipple [3] in open bile fistulas. These bile fistula dogs were fed liver in their diet as it was found that this prevented the intoxication so common in the open bile fistulas. No cod liver oil was given to these dogs.

More recently, as Whipple and Robscheit-Robbins [4] developed their program to study hemoglobin regeneration in anemic dogs, they observed this brown pigmentation of the muscle coats of the intestine in many dogs that had been fed liver over considerable periods of time. It was suspected therefore that this "maple sugar color" might be due to some dietary factor. The evidence points to *whole liver* in the diet as responsible for the deposit of this pigment in the cytoplasm of smooth muscle cells of the outer and middle muscle coats of the intestine. These observations have a bearing on the origin of pigment deposits of similar nature observed in human disease and old age.

EXPERIMENTAL OBSERVATIONS

Material was available for study coming from a variety of dogs kept anemic by bleeding at a level of 40 to 50 per cent hemoglobin, or one third normal for these dogs. The general care of these ani-

* Received for publication November 29, 1932.

mals, dietary régime and uniformity of living conditions have been described in detail in various publications from this laboratory.[4] As a rule these dogs are fed during control periods a *salmon bread* which among other things contains cod liver oil. This bread is a complete diet and its preparation has been carefully described elsewhere.[4] Various food factors may replace or supplement the basal ration of salmon bread and the amount of regenerated hemoglobin and red cells is determined by bleeding the surplus to reduce the dog to the usual anemic level of 40 to 50 per cent hemoglobin. A good many periods of liver feeding occur in the history of these dogs, as careful standardization on liver feeding is done over and over again.

Dog 24–90, with an Eck fistula, had been anemic for approximately 6 years. Age at death 8 years. During the long anemic period in this dog he was given many liver diet periods — a total intake of 26 kilos of various liver material. His liver was injured by chloroform and gradually he lost the capacity to make hemoglobin. He was killed and autopsied at once. The pigmentation of the muscle coats of the small intestine was well established. The stomach and colon showed much less pigmentation in the muscle coats. The pancreas was white. This material was used in the detailed study of this pigment recorded below.

Dog 25–97, a regular member of the anemia colony, was born in our kennels and after weaning was raised on a diet consisting of approximately 50 per cent cooked pig liver and 50 per cent salmon bread plus a milk powder. When full grown the dog was made anemic by bleeding and maintained at a level of 40 to 50 per cent hemoglobin. The usual control periods of salmon bread diet alternated with other diet factors, often liver or liver fractions. This dog in all ingested not less than 36 kilos of pig liver and probably more. The dog lived 5 years and 10 months. The anemia period lasted 5 years. Dog killed and autopsied at once. Pigmentation was a definite brown or buff in the muscle coats of the duodenum. Color faded in the lower small intestine and colon. The muscle coats of the stomach showed definite pigmentation which was much less marked than in the duodenum. This material was used in the tests recorded below.

Dog 30–119 was born in our kennels and after weaning was raised on a diet consisting of approximately 50 per cent cooked pig liver and 50 per cent salmon bread. To the daily diet mixture was added

10 to 15 cc. cod liver oil and 40 gm. whole milk powder. This diet régime lasted 10 months. When full grown the dog was made anemic by bleeding as usual and maintained at a hemoglobin level of 40 to 50 per cent for 7½ months. Age at death 19 months. Total food intake of liver amounted to 21 kilos. Laparotomy was done 3 months before death to fix the spleen to the abdominal wall. At this time it was noted that the small intestine presented a dark brown pigmentation. At autopsy 3 months later the same degree of brown pigmentation was recorded, although no liver had been given in the diet in this interval and the anemia had been continuous. This dog showed no pigmentation of the muscle coats of the stomach or colon. The pancreas was white. This material was used in the tests described below.

Dog 30–118, a litter-mate of Dog 30–119 above, was raised on a diet consisting of approximately 50 per cent raw hamburg steak and 50 per cent salmon bread plus 15 cc. cod liver oil and 40 gm. milk powder. The dog was on this diet 14 months and was anemic at the usual level for 7 months. At autopsy the dog showed no trace of pigment in the intestinal coats, either in gross or in histological preparations.

Dog 30–120, a litter-mate of Dog 30–119 above, was raised on a diet of salmon bread up to 300 gm., milk powder 40 gm. and cod liver oil 15 cc. The dog was on this diet for 14 months and was anemic at the usual level for 5 months. At autopsy the gastro-intestinal tract showed no trace of this brown pigmentation, either in gross or in histological preparations.

Dog 27–232 after weaning was raised on a daily diet consisting of salmon bread up to 300 gm., whole milk powder 40 gm., 15 cc. cod liver oil and a salt mixture 2 to 3 gm. The total iron intake was 35 to 40 mg. as Fe daily. The dog was in good health up to a day or so before death. The dog was never made anemic. Found dead. Autopsy showed the cause of death to be infestation with ascaris — the small intestine being full of round worms. The small intestine showed definite pigmentation of the muscle coats. This color was a pale buff or light brown, much less than in the dogs fed whole liver, but the color was definite and not to be mistaken. The salmon bread contained 7 cc. cod liver oil per 300 gm. in addition to the 15 cc. given daily.

Gross Appearance of Pigment

This pigmentation occurs mainly in the small intestines, shining through the unpigmented serous layer of the intestine, and is apparent immediately upon opening the abdomen. The muscle coats seem to be the only layers involved and the coloration appears to be confined to this particular structure. The mucosa is normal. The pigmentation is usually more concentrated in the upper portion of the small intestine, but occurs also in the large intestine, although here it is of a much lighter tone. The wall of the stomach sometimes shows a faint brown tint. Occasionally the pancreas is pigmented. The pigmentation varies from a light buff to a deep brown, often described as a "maple sugar color." This pigmentation of the intestinal tract is not accompanied by a hyperpigmentation of liver and kidney.

General Microscopic Description

Sections of the intestinal tract show that the pigment is found in both the internal and the external muscle layers, but it does not occur in the muscularis mucosae. It appears that occasionally the internal muscle coat contains a greater concentration of this pigment than the external. The color of the pigment is yellowish brown. The pigment is found in small granules in the cytoplasm. In cases of heavy pigmentation the cytoplasm appears densely packed with these granules. If, however, small amounts are found, the granules occur densely packed around the nuclei and rather sparsely in the periphery. These pigment granules generally are of uniform size in the same muscle cell and in unstained frozen sections may appear refractile. Occasionally these granules are found in such a fine form as to be visible only with high power magnification.

Reactions to Chemicals and Dyes

It has been observed by Whipple that this pigment is partially soluble in 95 per cent alcohol. Section material placed in alcohol gives up a portion of this pigment to this fluid medium. This solubility may vary, however, in different cases and may be independent of pigment concentration. For example in one animal (Dog 30–119) which showed heavy pigmentation only a small amount was soluble in alcohol, since the fluid was rather light in color even after a period

of 3 months. In some cases showing a less heavy pigmentation the tissues when placed in alcohol gave up a greater portion of the brown color, resulting in a highly colored solution demonstrating varying solubility of this pigment material. This was evident also in paraffin sections, since in some cases of a well marked gross pigmentation sections showed only slight pigmentation. In one case when sections were prepared from tissues kept in 95 per cent alcohol for a period of two months, these sections showed only a slight difference from those prepared from material fixed in formalin. Frozen sections from formalin-fixed material of the same case were made and placed in alcohol in order to determine whether solubility might be increased in a thinner layer of material. These sections were examined at different intervals for a 24 hour period but only a slight fading of the brown color was observed.

Other fat solvents such as ether, chloroform and xylol were tested on sections. Granules were uninfluenced but occasionally a slight amount of color was given off.

Iron reactions were negative in all cases (Berlin blue, Turnbull's blue reactions).

Various fat stains and lipoid reactions were tested (Sudan III, scharlach R, Ciaccio, Lorraine-Smith-Dietrich). Results were negative.

Concentrated Sulphuric Acid: The usual yellowish brown color immediately (seconds) becomes much darker and progressively becomes intensified during a 2 hour period. Following this some fading gradually occurs but the original light tones are not attained. Color change is more apparent in frozen sections. (Difference due to previous alcohol treatment of paraffin sections?) No blue coloration on adding acid is noted.

Concentrated Nitric Acid: 20 to 40 minutes following addition of this acid pigment shows slight bleaching. After 2 hours the granules become smaller and partially dissolve. Two to 3 hours later there remain only some undissolved granules in powder form.

Concentrated Hydrochloric Acid: No change following several hours.

Alkali (*50 Per Cent Sodium Hydroxide*): Granules swell during first 2 hours. Granules in the frozen sections become more refractile. Later the majority of the granules dissolve and there remains only a slight, diffuse brown tint.

Levaditi's Silver Nitrate Method: Granules become dark brown, some black. There are marked differences in different cases. Dog 24–90 showed moderate change, Dog 30–119 numerous black granules. Bielschowsky's method gave the same result.

Equal Parts 1 Per Cent Potassium Ferricyanide and 5 Per Cent Ferric Chloride: Granules become deep blue. The reaction is well marked only in frozen sections.

Hydrogen Peroxide (3 Per Cent and 5 Per Cent Solutions): Two to 3 hours slight bleaching. Fourteen to 16 hours later (observed at intervals) bleaching is more conspicuous — granules are not dissolved but shrunken. After 24 hours bleaching is marked but incomplete and granules are mostly dissolved.

Mounted sections and tissues were exposed daily for several hours to direct *sunlight* during a 5 day period. No effect on pigment.

Basic Fuchsin: In applying the iron reaction to these sections and using Mallory's basic fuchsin solution as counterstain it was noticed that these pigment granules take up the fuchsin. Paraffin sections give satisfactory staining results with basic fuchsin as employed by Mallory to demonstrate *hemofuscin*. For satisfactory staining of the pigment in frozen sections it is necessary to dilute the original basic fuchsin solution approximately 30 times. Staining with dilute solution for 2 to 3 minutes followed by 50 per cent alcohol to differentiate sections mounted in glycerine shows a varying intensity of staining in these pigment granules. Some partially decolorized when in alcohol show the original yellowish brown tint.

Neutral Red (1 Per Cent Aqueous Solution): Staining of frozen sections 2 to 3 minutes (for paraffin sections 8 to 10 minutes) gives an intensive red staining of granules. Sections were rinsed in water after staining.

Brilliant Cresyl Blue (0.5 Per Cent Aqueous Solution): Pigment granules stain intensely deep blue in contradistinction to paler blue with some violet tint of the nuclei. Staining of the granules fades somewhat within 2 to 3 weeks.

Nile Blue Sulphate (Saturated Aqueous Solution): Pigment stains intensely a dark blue, similar in tint but more intense than nuclei. No pinkish violet tint of neutral fat in the granules.

Methylene Blue: Granules show some greenish tint. Probably not a true stain but an admixture of dye and native yellowish brown color of the pigment.

Discussion

Such pigmentation in animals has not been mentioned by other investigators so far as we know. Mallory and coworkers [5, 6] however observed in experiments with copper administered to rabbits, orally or parenterally, pigment especially in the heart, liver and kidneys which seems not unlike the pigment in these dogs. Mallory compares this pigment to that found in human cases of hemochromatosis. He believes that the pigmentation of his copper-fed animals is identical with the pigment of hemochromatosis. He calls this pigment *hemofuscin* (the name *hemofuscin* was first applied by von Recklinghausen in cases of hemochromatosis) and suggests that it is an intermediate stage between hemoglobin and hemosiderin. Finally it becomes *hemosiderin* in some organs but generally is not found as such in the smooth muscle.

As to origin, classification and nomenclature of this iron-free pigment observed in human hemochromatosis and in old age there exists considerable difference of opinion.

Hueck [7, 8] names it lipofuscin and believes that the pigment bears a relation to fats and fatty acids and differentiates them sharply from melanins. He based his differentiation upon dissimilarities of staining and microchemical reactions mainly upon the fact that this so-called lipofuscin takes on the fat stains, whereas melanin takes no such stain. Furthermore lipofuscin does not bleach readily with hydrogen peroxide, whereas melanin does.

Lubarsch,[9] Brahn and Schmidtmann [10,11] do not differentiate sharply between melanin and lipofuscin, as well as hemofuscin. They believe that all this pigment belongs to the melanin group because they were unable to find sharp differences between chemical reactions as described by Hueck.

Connor [12] investigated the pigmentation of old age, which he calls hemofuscin. His description of the various staining and chemical reactions of this pigment corresponds closely to that found in our dog material. He does not mention the partial solubility of the pigment in alcohol.

Based on the similarity of certain reactions it seems to us that the so-called hemofuscin of Connor, as well as the lipofuscin of Hueck, belong to the same group and may represent a pigment complex.

The dissimilarities of the different chemical and staining reactions may signify a different grouping of radicles within the same complex.

The microchemical and staining reactions of the pigment found in our dogs create an impression that it might be similar to the above mentioned group and represent a pigment complex composed of groups rather than single pigment entities. Our own data and differences observed in different animals as to these various reactions (solubility in alcohol, silver nitrate reaction, fuchsin staining) would strengthen the belief in a pigment complex. The results of microchemical and staining reactions indicate that the pigment found in our dog material may be related to the pigment found in the human material cited above. There is also a similarity in the localization of these pigments in the intestinal tract of our dogs and of human cases which show this pigmentation in the intestine.

Mallory and Connor suggest that this hemofuscin pigment is related to hemoglobin disintegration. In our cases this cannot be admitted and the relation of this pigmentation to diet intake seems established.

We have been unable to convince ourselves that this pigment is related closely to the fats or fatty acids as Hueck claims for lipofuscin.

Our experimental data show that this pigmentation of the muscle coats of the gastro-intestinal tract in dogs is found in anemic and non-anemic dogs, in bile and Eck fistulas, in young and old dogs. One thing alone is constant — that is a large food intake of whole liver or cod liver oil. Whole liver feeding gives a deep pigmentation, while cod liver oil feeding alone may or may not cause a faint pigmentation of the small intestine (Dogs 30–120 and 27–232 above). It will be of interest to observe whether this pigment is found in other animals fed liver and liver fractions. Some pathologist may have opportunity to observe human cases where a prolonged diet intake of liver or liver fractions has preceded death.

Summary

A peculiar brown or buff pigmentation of the muscle coats of the intestinal tract has been observed in dogs. Some of these dogs had bile or Eck fistulae with or without anemia. Other dogs were in the anemia colony of this laboratory and had been continuously anemic

from bleeding for various periods of time. Other dogs were normal. Age was not a factor.

Microchemical reactions and staining properties of this pigment are given. On the basis of these tests one observes some similarity to the pigment observed in human disease (hemofuscin) and in old age.

It is suggested that this pigment is not a definite entity but perhaps a pigment complex. The experiments indicate that this pigment is of dietary origin due to some liver constituent which is absorbed.

Observations on human cases of anemia where large amounts of liver or liver fractions have been administered should prove of interest.

REFERENCES

1. Whipple, G. H., and Hooper, C. W. Bile pigment metabolism. VII. Bile pigment output influenced by hemoglobin injections, anemia and blood regeneration. *Am. J. Physiol.*, 1917, **43**, 258–274.
2. Hooper, C. W., and Whipple, G. H. Bile pigment metabolism. VIII. Bile pigment output influenced by hemoglobin injection; splenectomy and anemia. *Am. J. Physiol.*, 1917, **43**, 275–289.
3. Wisner, F. P., and Whipple, G. H. Variations in output of bile salts and pigments during 24 hour periods. *Am. J. Physiol.*, 1922, **60**, 119–133.
4. Whipple, G. H., and Robscheit-Robbins, F. S. Blood regeneration in severe anemia. I. Standard basal ration bread and experimental methods. *Am. J. Physiol.*, 1925, **72**, 395–407.
5. Mallory, F. B., Parker, F., Jr., and Nye, R. N. Experimental pigment cirrhosis due to copper and its relation to hemochromatosis. *J. Med. Res.*, 1920–21, **42**, 461–490.
6. Mallory, F. B. The relation of chronic poisoning with copper to hemochromatosis. *Am. J. Path.*, 1925, **1**, 117–133.
7. Hueck, W. Pigmentstudien. *Beitr. z. path. Anat. u. z. allg. Pathol.*, 1912, **54**, 68–74.
8. Hueck, W. Die pathologische Pigmentierung. Handbuch der allgemeine Pathologie, Krehl, L., and Marchand, F. S. Hirzel, Leipzig., 1921, **3**, Pt. 2, 298–481.
9. Lubarsch, O. Über das sogenannte Lipofuscin. *Virchows Arch. f. path. Anat.*, 1922, **239**, 491–503.
10. Brahn, B., and Schmidtmann, M. Pigmentstudien: Zur Kenntnis des Melanins und des braunen Abnutzungspigments. *Virchows Arch. f. path. Anat.*, 1919–20, **227**, 137–152.
11. Brahn, B., and Schmidtmann, M. Zur Pigmentfrage. *Virchows Arch. f. path. Anat.*, 1922, **239**, 488–490.
12. Connor, C. L. Studies on lipochromes. IV. The nature of the pigments in certain organs. *Am. J. Path.*, 1928, **4**, 293–308.

STRUCTURE SUGGESTING A SPINAL CORD FOUND IN AN OVARIAN DERMOID *

JOSEPH MCFARLAND, M.D., SC.D.

(From the McManes Laboratory of Pathology, University of Pennsylvania, Philadelphia, Pa.)

A year or more ago it was necessary to prepare a class set of microscopic sections, showing the essential structure of the ovarian dermoid, for students to study in the course of instruction in histopathology. Through the kindness of friends and assistants a number of blocks of tissue from various sources were obtained, and after sample sections had been made from them, the one herein to be described was selected. The material is thought to have come from the collection of Dr. Charles C. Norris in the University Hospital, but, as at the time of its collection it was not suspected that reference to the gross material or records would ever be necessary, no identification was kept. Under these circumstances, attempts to trace the block of tissue to its original source have failed, and future attempts seem hopeless.

The material consisted of one block of tissue cut from the "dermoid plug." On each side, and probably on the top, it showed shaggy hairy skin, while the bottom was a smoothly cut surface. Through the center of the block, and passing from end to end, there was a slender cylinder, the length of which cannot now be determined as it had been cut off transversely at each end. It is concerning the nature of this cylinder that we are about to speculate.

When cut sections of the block are examined, the microscopic structure is found to bear out the gross appearance. Skin, with large papillae, hair follicles, sebaceous and sweat glands, is found to make up the greater part, and the whole would have been commonplace had it not been for the presence and peculiar qualities of the cylinder mentioned above, which is composed of nervous tissue.

In describing the "dermoid plug," Frank, in his Gynecological and Obstetrical Pathology, 1931, page 401, says: "On inspection of the gross sections, or with a very low magnification, a certain uniformity of arrangement will become apparent. In the most typical

* Received for publication December 2, 1932.

cases the skin region corresponds to the scalp. Below this, within a fibrous capsule, often reinforced by bone plates, is the brain (pigmented area for eye anlage). In close conjunction are found bone masses with teeth."

The position of the nervous tissue in our section corresponds to that of the "brain" mentioned by Frank, and indeed, it may be a "brain," but its elongated cylindrical form is more suggestive of spinal cord. There are no bony plates homologous with cranial bones, nor is there any area of pigmentation representing the anlage of the eye. The nervous tissue lies in such close juxtaposition to the skin that the hair bulbs appear almost to connect with the membranes by which it is surrounded, as shown in Figure 1.

The configuration of the transverse section of the nervous tissue is strikingly like that of the spinal cord and it is surrounded by a somewhat dense pia arachnoid, although there is no separate dura. There are no definite anterior and posterior commissures, but at several points there is a deep penetration of the membranes, with division of the nervous tissue more suggestive of the commissures of the spinal cord than of the sulci of the brain. There is no central canal, but almost in the center there is a rounded pale mass of neuroglia that may have resulted from abnormal proliferation of ependymal cells.

Under a higher power, the greater part, indeed almost all, of the nervous tissue is found to consist of gray matter, that is, neuroglia containing nerve cells, the only part suggesting white matter being near the center. This arrangement is more like that of the brain with its cortex of gray matter than that of the spinal cord with its H-shaped center of gray matter and peripheral tracts of white matter; but at numerous marginal areas the neuroglia is loose and open, forming a kind of reticulum, as though prepared for penetration by axones, such as would have provided the various tracts of white matter had development progressed along normal lines. Here and there, in the general mass, areas of medullated nerve fibers are observed, but never with such definiteness as to constitute a column or tract. The paucity of white matter might be explained on the assumption that few axones have been formed in the absence of members to be supplied or functions to be performed. Failure of the white matter to develop would also account for the minute size of the cord. The nerve cells are clustered ganglion-wise here and

there, but nothing definite like the columns of Clark appears. Many of the cells give off visible axones.

It is by no means impossible that this nervous tissue is but an unusual form assumed by the "brain" of the "dermoid plug," but its elongated cylindrical form, its appearance in transverse section, its suggestion of commissures and the clusters of ganglion cells, unlike what is seen in the cerebral cortex, suggest that in this case the "brain" has developed in the form of a spinal cord.

In J. Veit's Handbuch der Gynäkologie, 1908, **4**, Pfannenstiel edits a section, "Die Erkrankungen des Eierstockes und des Nebeneierstockes," of which P. Kroemer, of Berlin, contributes a section entitled "Die Dermoidkystome." On page 230 of this work appears an illustration of a section through a spinal cord that forms part of the structure of an ovarian dermoid. It has surrounding membranes, an elongated, slightly asymmetrical form, a large central canal lined with a single layer of beautiful columnar ependymal cells, and in close relation to it is a large sympathetic nervous ganglion into which a large medullated nerve enters. This is the only occurrence of "spinal cord" in an ovarian dermoid that seems to have been reported.

The probability of the structure observed by Kroemer being a spinal cord is greatly increased by the presence of the central canal, and by the juxtaposition of the nerve ganglion, and that of our case diminished by the absence of both. But a sympathetic ganglion might occur equally well in juxtaposition to brain or spinal cord so that it is not an infallible means of identification, and the large size of the supposed central canal in Kroemer's case might almost as well be interpreted as a ventricular cavity.

It is well known that the various structures in the same dermoid cyst show remarkable inequality of development, some being adult while others are embryonal, and that they are not infrequently overtaken by pathological changes in the course of development. In Kroemer's case the development of the nervous tissue is more advanced, in ours less so; while in ours in the position where the central canal should have appeared, a pathological gliosis seems to have suppressed it.

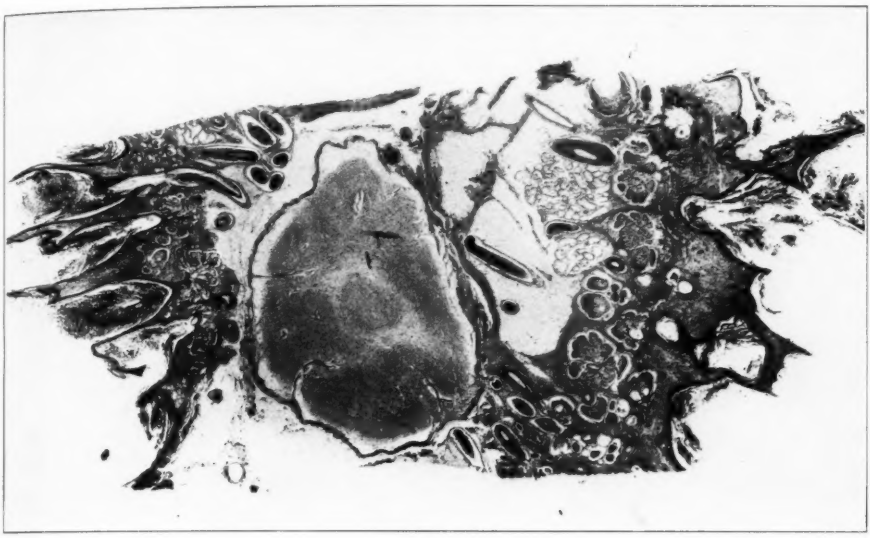
The difficulty of identification is too great to warrant a definite decision, but the probability that the structure found in our sections is a spinal cord seems sufficient to justify its being recorded as such.

DESCRIPTION OF PLATE

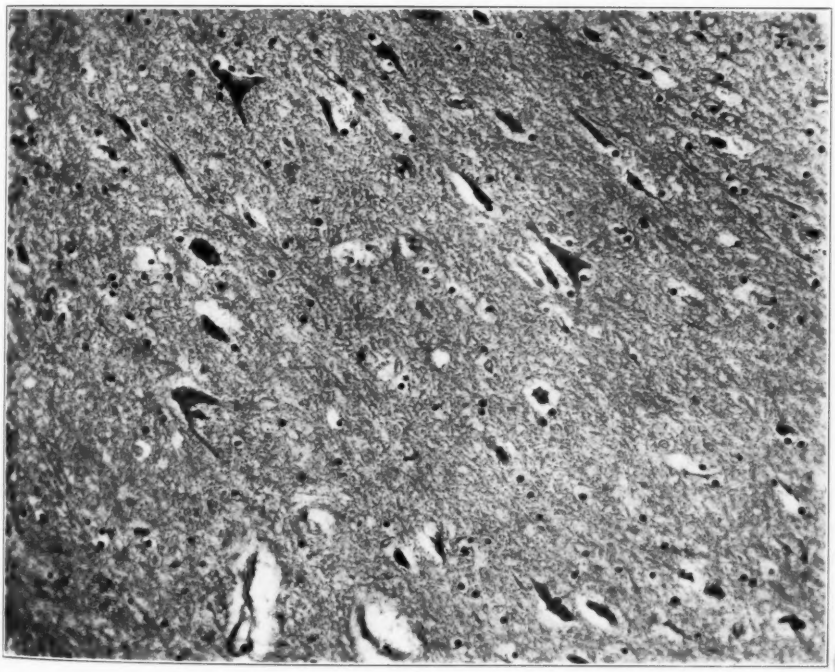
PLATE 37

FIG. 1. General low power view showing the section of the supposed spinal cord surrounded by its membranes.

FIG. 2. Area of a part of the section showing the multipolar ganglion nerve cells, some of which show growing axones.



1



2

McFarland Structure Suggesting Spinal Cord